THE CELL-MEDIATED IMMUNE RESPONSE IN EPIDERMAL DEFENCE AGAINST *DERMATOPHILUS CONGOLENSIS*

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This presentation is entirely the product of my own efforts and the work on which it is based was my own except where specifically stated in the text and in the acknowledgements section.

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EQUATIONS

ABBREVIATIONS

Ag	antigen
AMP	adenosine monophosphate
APC	antigen-presenting cell
B/A	blood agar
BHI	brain-heart-infusion
BSA	bovine serum albumin
Con A	concanavlin A
CPM	counts per minute
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
DNCB	1-chloro-2,4-dinitrobenzene
DPM	disintegrations per minute
DTH	delayed-type hypersensitivity
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ETAF	epidermal cell-derived thymocyte activating factor
FITC	fluorescein isothiocyanate conjugate
FCS	foetal calf serum
HBSS	Hank's balanced salts solution
Ia	immune-associated
IFAT	indirect fluorescent antibody test
IFNγ	gamma interferon
Ig	immunoglobulin
IL-1	interleukin one

LTT	lymphocyte transformation test
M199	medium 199
MC	mononuclear cell
MEM	minimum essential medium
MHC	major histocompatibility complex
MMIF	macrophage migration inhibition factor
NRS-BSA	normal rat serum-bovine serum albumin
NSP	neutralised soya-peptone
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PMN	polymorphonuclear leucocyte
PPD	purified protein derivative of *Mycobacterium tuberculosis*
psi	pounds per square inch
PWN	pokeweed mitogen
RPMI	Rosewell Park Memorial Institute medium
SALT	skin-associated lymphoid tissue
SAM	sheep anti-mouse
SI	stimulation index
SMC	spleen mononuclear cell
SMEM	suspension-minimum essential medium
TNFα	tumour necrosis factor alpha
UV	ultra-violet

ABSTRACT

This study centred on the host cell-mediated immune response to experimentally-induced infection with *Dermatophilus congolensis*. A review of the literature explored the characteristics of both chronic and acute infections and, in particular, the host response. Defence mechanisms of the skin were also considered.

Spleen mononuclear cells were isolated from experimentally-infected Wistar rats and cultured with a range of doses of inactivated *D.congolensis* cocci. The outcome was assessed with a lymphocyte transformation test. Mononuclear cells from infected rats responded well and in a dose-dependent fashion to *in vitro* stimulation, with proliferation rates which were significantly greater than those of controls ($P < 0.01$). The supernatants of the stimulated mononuclear cells were assayed for lymphokine activity and were found to cause significant inhibition of macrophage migration ($P < 0.05$).

The immunophenotype of the responding population was determined with an indirect fluorescent-antibody test using a range of anti-rat mononuclear cell monoclonals. A significant increase occurred in the T-helper population of cells from infected rats to form 56 per cent of the total in the presence of an optimal concentration of *D.congolensis* cocci ($P < 0.05$).

The *in situ* host response to *D.congolensis* was studied by identifying the immunophenotypes within the cellular infiltrate which collects at an infection site. Sections of skin were screened with a range of anti-rat monoclonal antibodies. Initially, polymorphonuclear leucocytes predominated but were soon superseded by a T-cell infiltrate. Both T-helper and T-cytotoxic/suppressor cells were present and were in an activated state, demonstrated by the expression of interleukin-2 receptor and class II. The T-helper cells were observed in the dermis and the epidermis, whereas the T-cytotoxic/suppressor cells were only rarely seen in the epidermis. In common with some other skin diseases, the epidermis expressed class II antigens.

Epidermal hyperproliferation may be an important defence mechanism against pathogens such as *D.congolensis*. Several different types of epidermal culture system were first investigated to assess their suitability as indicators of epidermal proliferation. Low calcium concentrations induced stable, monolayer cultures whereas cultures under normal calcium conditions were inherently unstable, although the cells grew well short-term. Both types of culture system were then used to determine whether stimulated mononuclear cells might play a role in epidermal defence by causing hyperproliferation. Thus, culture supernatants from *D.congolensis*-stimulated mononuclear cells were added, at various dilutions, to the epidermal cultures and the cell proliferation rates assessed with a ^{3}H-thymidine incorporation assay. The supernatants caused either significant inhibition or stimulation of proliferation ($P < 0.01$), the net effect being concentration-dependent. In contrast, culture supernatants derived from naive rats had no stimulatory effect on the proliferation of rat epidermal cells; but at the highest concentration caused significant proliferation inhibition ($P < 0.01$).

The results were discussed in relation to the likely immune effector mechanisms responsible for the rapid resolution of experimentally-induced infections and the possible defects in this response which allow progression to the generalised, chronic form of the disease.

INTRODUCTION

Dermatophilosis is an exudative dermatitis, commonly affecting cattle, sheep and horses and caused by the ubiquitous bacterium *Dermatophilus congolensis* (Stewart 1972a). Acute and chronic syndromes occur, the latter being significantly more serious and geographically restricted to cattle in West and Central Africa (Hyslop 1980) and the Caribbean (Burridge 1985) and to sheep in Africa (Macadam 1977) and Australia (Bull 1929). The reason for the occurrence of a mild infection in one situation and a severe one in another is unknown; however, several external factors have been implicated in contributing to the progression of the disease to a chronic, generalised state (Oduye 1976a).

Little is known of the host immune response to infection; a humoral response does develop but it is not protective (Richard, Thurston and Pier 1976). A cell-mediated immune response also occurs being characterised by the development of a delayed-type hypersensitivity (DTH) reaction and a localized infiltration of mononuclear cells. Although immunity *per se* does not develop, accelerated healing occurs on re-infection and is associated with the onset of the DTH response (Roberts 1966a).

To date, there are no published studies on the immunophenotype of the cells which infiltrate the skin at the site of *D.congolensis*-infection, except for one report which identified immunoglobulin A-bearing plasma cells amongst the leucocytes (Ellis, Robertson, Sutherland and Gregory 1987). Likewise, little is known about which lymphoid cells may participate in the host protective immune response.

Dermatophilosis is a disease normally confined to the epidermis (Amakiri 1974) and, following an infection, the epidermis displays marked

abnormalities such as pronounced thickening (Oduye 1976b). The acanthosis is suggestive of either increased epidermal proliferation or of an increased transit time leading to decreased cell shedding. The occurrence of parakeratosis (Bida and Dennis 1977), which indicates incomplete differentiation, favours the former. Furthermore, acanthosis is a common feature of conditions where the epidermis has a high turnover rate (Bullough 1972). Epidermal hyperproliferation, with an increased desquamation rate, may be an important defence mechanism against some skin infections (Berk, Penneys and Weinstein 1976). Mononuclear cells infiltrate the skin in response to the presence of *D.congolensis* (Schulz 1955) and may co-operate with the epidermal cells, by means of soluble mediators, in hindering further invasion. Indeed, epidermal cells release cytokines which may attract and activate mononuclear cells (Luger, Kock, Danner, Colot and Micksche 1985); these, in turn, release lymphokines which may induce epidermal hyperproliferation (Ristow 1987).

This study examines aspects of the host immune response to experimentally-induced *D.congolensis*-infection with a view to identifying the likely defects in the response to a natural infection. Such defects may allow progression to the chronic, generalised and economically important form of the disease.

CHAPTER ONE

LITERATURE REVIEW

CONTENTS (cont.)

DERMATOPHILOSIS

Dermatophilosis is predominantly a disease of domestic ruminants, with cattle and sheep being the most commonly affected; horses are also susceptible. In addition, *Dermatophilus congolensis* has been isolated from animals as diverse as zebras (Green 1960) and polar bears (Smith and Cordes 1972). *D.congolensis* infection in man has occasionally been reported (Albrecht, Horowitz, Gilbert, Hong *et al* 1974) and is considered by some to be a true zoonosis. However, only a handful of people are known to have become infected and in all cases symptoms were very mild (Kaplan 1976).

ECONOMIC IMPORTANCE

The economic losses caused by dermatophilosis vary according to the severity and geographic distribution of the disease. Thus, dermatophilosis is of little economic significance in temperate countries where it occurs infrequently and where infected animals normally develop a mild form of the disease (Lloyd 1976). In contrast, dermatophilosis is relatively common in the tropics and is typically characterised by generalised, chronic lesions.

The most obvious economic losses result from damage to hides and downgrading of wool (Austwick and Davies 1958). The hides industry in Nigeria has been singled out for suffering large financial losses due to the disease (Bida and Dennis 1976). Bovine dermatophilosis is regarded as one of the most important diseases limiting improved cattle production (Ilemobade 1984) and draught animal efficiency in Nigeria (Lloyd 1976). Indeed, the disease has restricted the introduction of potentially

productive exotic cattle breeds to West and Central Africa due to their high susceptibility (Lloyd 1976).

Mortality is generally low in indigenous breeds, except when animals are stressed by malnutrition and the adverse climatic conditions which occur at the onset of the rainy season. Dermatophilosis and other persistent infections are often fatally exacerbated under these circumstances (Plowright 1956, Hyslop 1980). In contrast, mortality in exotic breeds may be high (Stewart 1972a).

CLINICAL SIGNS

The typical lesion in cattle is an exudative, hairless, crust-like thickening of the epidermis. Lesions begin as small papules which exude and then coalesce to form extensive, adherent scabs (Figures 1.1 and 1.2, Stewart 1972a). The symptoms in sheep are also those of a proliferative and exudative dermatitis with bundles of wool fibres matted together by exudate (Bull 1929).

In severe cases, lesions can spread to cover a large part of the body surface. Often lesions are localised at certain sites; these vary, but back, neck, head and leg lesions are common (Bida and Dennis 1976). Other characteristics include decreased milk yields and a loss in general condition (Lloyd 1976).

CLASSIFICATION OF *DERMATOPHILUS CONGOLENSIS* AND LIFE CYCLE

Dermatophilus congolensis was named and first described by Van Saceghem (1915). He thought the organism was a fungus and this belief continued for some time. Further confusion has arisen with the many different names applied to both the organism and the disease throughout the years.

Dermatophilus congolensis is classified according to the following scheme (Stewart 1972b):

Class: *Schizomycetes*

Order: *Actinomycetales*

Family: *Dermatophilaceae*

Genus: *Dermatophilus*

Species: Congolensis

In common with other actinomycetes, *D.congolensis* is a Gram-positive, branching, filamentous bacterium. However, *Dermatophilus congolensis* is characterised by an unusual growth form whereby its filaments are composed of multiple rows of cocci (Figure 1.3).

Filaments arise as extensions of germ tubes from cocci and will grow for several micrometers before branching and formation of septa. Firstly transverse and then longitudinal septa form, giving rise to parallel rows of individual cells. These are encapsulated by a gelatinous matrix, which arises from thickening of the cell walls. Dissolution of the matrix results in liberation of motile cocci, which are about 0.5-1.0 μm in size (Gordon and Edwards 1963). Each of these cells can possess up to fifty flagella, which form either before or after liberation (Richard, Ritchie and Pier 1967). The cell cycle is completed when the motile cocci germinate after reaching a favourable site. Roberts (1963b) demonstrated a chemotactic response of the motile cells towards carbon dioxide. He thought this might aid infection since carbon dioxide is released from the skin and therefore may attract *D.congolensis* cocci.

GROWTH CHARACTERISTICS

Dermatophilus congolensis grows well on blood agar and in certain fairly complex broths such as brain heart infusion (BHI) containing serum, or a 50/50 mixture of BHI and neutralised soya peptone.

Culture morphology is extremely variable with irregular, raised colonies and colours ranging from whitish grey to orange. The optimum growth temperature is 37°C and growth is enhanced by an elevated carbon dioxide concentration (Roberts 1963c). The nutrient source *in situ* is unknown. Roberts (1965a) believed *D.congolensis* is unable to breakdown and utilise keratin, whereas Isitor (1984) stated the opposite, noting marked regions of keratolysis surrounding the bacterium. It seems likely that it utilises the proteins and other substances present in the cellular exudate which collects at the site of infection.

TRANSMISSION

The question of how dermatophilosis is transmitted is so far unresolved. Transmission does not appear to result from simple animal-to-animal contact since non-infected cattle can be grazed together with clinical cases without contracting the disease (Plowright 1956).

The occurrence of dermatophilosis is often associated with the presence of the tick *Amblyomma variegatum* (Plowright 1956). Macadam (1962) claimed to have achieved direct transmission of dermatophilosis with the tick as vector but his conclusion was based on the result from one experimental animal. He noted development of localised paper-thin scabs on one rabbit after allowing ticks, taken from naturally-infected cattle, to feed on its ear. Later, Oppong (1976) isolated *D.congolensis* from *Amblyomma variegatum* ticks taken from naturally-infected Ghanian cattle.

Richard and Pier (1966) transmited *D.congolensis* from donor to

recipient rabbits via the stable fly, *Stomoxys calcitrans*, and the house fly, *Musca domestica*. Sixty-five per cent of transmission attempts with *S.calcitrans* resulted in the development of characteristic lesions providing both the donor and recipient sites had been pre-wetted. *D.congolensis* was isolated from *M.domestica* and from the legs of *S.calcitrans* but not from its mouthparts despite the fact that these cause mechanical disruption of the skin. Philpott and Ezeh (1978) were unable to repeat these transmission results with cattle except on one occasion. Other potential sources of infection such as contaminated soil are not thought to play a major role in the aetiology of the disease (Roberts 1963a).

Several modes of transmission of *D.congolensis* might well be possible; the organism may be an opportunist pathogen and a normal resident of the skin microflora (Zlotnik 1955). A number of predisposing factors are, however, required for development of generalised dermatophilosis and will be discussed later.

DIAGNOSIS

Dermatophilosis is diagnosed by examination of gross lesions, which have a characteristic appearance, and of smears made from the underside of the scabs. Microscopic examination of smears will demonstrate filaments composed of multiple rows of cocci which are typical of *D.congolensis* (Figure 1.3). Isolation of the organism into culture will confirm the diagnosis (Roberts 1967).

Occasionally, problems in identification are encountered; for example, when lesions are very old. The direct fluorescent-antibody test for *D.congolensis* developed by Pier, Richard and Farrell (1964) has proved useful, although this may be superseded by a test using the monoclonal

antibody to *D.congolensis* which was recently described (How and Lloyd 1988).

Antibody-detecting techniques used to assess dermatophilosis include agar gel precipitation, indirect haemagglutination and particulate antigen tube agglutination tests (Pulliam, Kelley and Coles 1967). Oduye (1974) and Aghamo and Lloyd (1983) investigated different *D.congolensis* antigen preparations to determine which was the most reliable for use in circulating antibody tests and both found concentrated culture supernatants to be the most effective source of antigen.

The limitation of such tests is that they can only show that an animal has at some time been exposed to *D.congolensis* and not necessarily that it has an ongoing infection. Lloyd (1981) found that Ayrshire cattle which had apparently never shown any clinical symptoms of the disease nevertheless possessed antibodies to *D.congolensis*. He was able to demonstrate this by use of an enzyme-linked immunosorbent assay developed by him and which he found to be more sensitive than previous antibody detecting methods.

TREATMENT AND CONTROL

Dermatophilus congolensis is highly sensitive to a range of antibiotics *in vitro* (Plowright 1958, Vanbreuseghem, Takashio, El Nageh, Presler *et al* 1976). However, chronic cases of dermatophilosis are refractory to treatment because of the inaccessibility of the organism at the site of infection where it is protected from external agents by a thick layer of scab and, to some extent, from parenteral agents by virtue of the relative remoteness from the blood supply.

The traditional method of treatment is topical application of, for example, sulphur in groundnut oil (Bida and Dennis 1976). It is generally

agreed, however, that topical preparations are ineffective or of limited value (Ilemobade 1984). Nevertheless, Blancou (1976) claimed that mild cases of the disease may be cured with cresol or copper-based treatments.

In many areas the use of topical agents has been superseded by parenterally administered antibiotics. Ilemobade (1984) found a single, intramuscular dose of long-acting "Terramycin" (oxytetracycline) to be effective in treating naturally infected cattle. All animals with mild lesions were cured as were the majority of animals with a generalised infection. Sarradin, Akakpo, Bornarel, and Mohamadou (1985) also testified to the efficacy of long-acting "Terramycin" and to its superiority over the penicillin-streptomycin mixtures previously advocated (Blancou 1976). In addition, they found a significant increase in the cure rate when the antibiotic therapy was combined with weekly tick-dipping. Response rates to antibiotic treatment are extremely variable and are dependant on a number of factors such as the degree of infection. Sarradin et al (1985) reported cure rates of 89 per cent with long-acting "Terrramycin" but Marchot and Leroy (1987) obtained a cure rate of only 36 per cent with the same antibiotic at the same dose. Moreover, antibiotics are also relatively expensive as a form of treatment.

There has been much research into the possibility of developing a vaccine against dermatophilosis but results have been equivocal (Lloyd 1984). Chamoiseau and Lefevre (1973) induced protection against re-infection in rabbits intradermally vaccinated with a live culture of *D.congolensis*. Others have been unable to repeat this result (Bida and Kelley 1976, Aghomo and LLoyd 1983). Since animals recovering from a natural infection do not gain immunity to re-infection a vaccine is unlikely to prove to be efficacious (Bida and Kelley 1976, Hyslop 1980).

In view of the lack of a reliable and inexpensive form of treatment and the infeasibility of vaccination, at least at present, it would seem that efforts should be directed towards prevention rather than cure.

A consensus exists that dermatophilosis may be controlled to some extent by control of the tick population. Plowright (1956) carried out a field trial in Nigeria of the efficacy of dipping. Twenty cattle were kept undipped and another twenty were dipped twice weekly with "gammexane", a proprietary preparation of gamma-benzene hydrochloride (B.Vet.C.). After 15 weeks all the undipped cattle had developed natural infections, whereas all the dipped animals were free of lesions. Such spectacular results are not often repeated under normal field conditions, partly due to ineffective dipping procedures. Furthermore, Ilemobade (1984) found that even where a rigid tick control programme operated, the clinical disease still persisted, though with a reduced incidence.

In general, where there is good management and animals are kept under favourable conditions, the disease is not a problem but this is rarely achievable in most tropical countries (Lloyd 1984), hence the continued search for an effective and cheap method of treatment or control.

HISTOPATHOLOGY OF NATURAL INFECTION

The epidermis and the hair follicle sheath are the prime sites of invasion for *Dermatophilus congolensis.* Roberts (1965a) and Bida and Dennis (1977) believed that *D.congolensis* invaded only the living epidermis, the cells of which then appeared to become keratinised. However, most authors claim the organism is most commonly found in the *stratum corneum* i.e. the keratinised layer and is only rarely seen in the other epidermal layers (Zlotnik 1955, Amakiri 1974, Oduye 1975, Bwangamoi 1976, Isitor 1984).

Dermatophilus congolensis is often found in the hair papillae and sheath, but not in the hair shaft itself (Amakiri 1974). Zlotnik (1955) held the view that *D.congolensis* enters the skin via the hair follicles which then rupture, allowing filaments to spread out into the *stratum corneum*. Amakiri (1974) reported the occasional spread of filaments from ruptured follicles into the epidermis as far down as the basal layer. Further spread was apparently halted by the basement membrane. Any breaches in the basement membrane, however, were followed by limited growth of *D.congolensis* into the dermis. Nevertheless, the organism is only rarely seen in the dermis (Bwangamoi 1976) and Zlotnik (1955) never observed it below the epidermis. *D.congolensis* is also able to invade sebaceous glands (Amakiri 1974).

A cellular exudate collects in the *stratum corneum* in response to the presence of *Dermatophilus congolensis*. The exudate, which contains polymorphonuclear leucocytes (PMN), lymphocytes, eosinophils, plasma cells and macrophages, acts as a barrier to downwards spread of the filaments (Amakiri 1974). In the upper dermis, oedema and infiltration by PMN, lymphocytes and a few plasma cells takes place (Amakiri 1974).

Zlotnik (1955) found the lymphocyte to be the predominant cell type in natural infections of bovine skin and Schulz (1955) found it to dominate the dermal response. In contrast, Roberts (1965a), studying primary experimental sheep infections, found PMN were the most abundant and stated the same was true for natural infections. Mémery and Thiéry (1960) emphasized the distinction between different stages of infection and studied initial lesions in primary infections. They noted an inflammatory response in the dermis, followed by an epidermal response, with PMN dominant. It may well be that a non-specific, inflammatory response occurs during a primary infection; such that a polymorphonuclear

cellular reaction results. Whereas, in a previously exposed animal, or in a chronic case, the response is predominantly lymphocytic. This hypothesis is supported by the observations of Oduye (1976b) who found the dermal reaction was a polymorphonuclear one in early stages with few lymphocytes present. In contrast, in chronic cases, the response was lymphocytic. However, both types of cells were always present.

Examination of skin biopsies from cases of natural infection reveal changes in the cutaneous blood vessels. Amakiri and Nwufoh (1981) found a three-fold increase in the number of capillaries in the upper dermis of infected skin as compared to normal skin. The vessels were dilated and engorged with blood cells.

Infection with *D.congolensis* results in a hyperplastic epidermis together with other characteristic changes. Degeneration of some of the epidermal cells is manifested by oedema or necrosis (Schulz 1955). The epidermis becomes acanthotic, i.e. the *stratum spinosum* increases in thickness as does the *stratum corneum* (hyperkeratosis). At the same time parakeratosis occurs, i.e. differentiation is affected such that the cells of the *stratum corneum* retain their nuclei (Schulz 1955). Amakiri (1974) thought the acanthosis and hyperkeratosis occurred as a means of eliminating the invading organism. Zlotnik (1955) claimed the epidermal proliferation occurred as a result of stimulation by the surrounding lymphocytes.

Dermatophilosis is characterised by the presence of thick scabs composed of alternate layers of keratinised epidermis and cellular exudate that arise as a result of a cycle of invasion and epidermal response (Hyslop 1980). Once the epidermis becomes infected the exudate begins to separate it off from the dermis and a scab forms. Meanwhile, a new and hyperplastic epidermis develops below. In a chronic infection,

the newly-formed epidermis will also become invaded and the cycle repeats (Roberts 1965a).

EXPERIMENTAL INFECTION

Probably, the most important hindrance to the study of dermatophilosis is the inability to reproduce the natural, chronic disease by experimental infection and, despite numerous attempts, no satisfactory model of the natural disease exists (Mémery and Thiéry 1960, Oduye 1975, Abu-Samra, Imbabi and Mahgoub 1976a, Blancou 1976 and Lloyd 1984)

Dermatophilus congolensis appears to be unable to infect healthy, intact skin, therefore, to produce an experimental infection, the skin barrier must be damaged (Oduye 1976a). In the cow, the removal of the surface sebum layer with a solvent such as ether, after first clipping the hair from the site, suffices (Lloyd and Jenkinson 1980). Likewise, sheep skin may be prepared for infection by clipping and de-fatting with petroleum (Roberts 1965a). Alternatively, the integrity of the skin can be broken by superficial scarification, with or without prior de-fatting (Abu-Samra, Imbabi and Mahgoub 1976a). Once the skin barrier has been broken an experimental lesion will result after application of an inoculum of *D. congolensis*.

Unlike the natural chronic, spreading form of the disease, artificially-induced lesions are transient and never spread outside of the inoculation site (Figure 1.4). Typically, erythema of the site occurs 24 hours after experimental infection and by 48 to 72 hours a serous exudate is evident. Scab formation is detectable at this time and by 96 hours is well-developed. After about six days the scab begins to detach and has usually disappeared by day ten (Oduye 1975). Limited success in inducing a chronic lesion was achieved by Davis and Philpott (1980).

They were able to prolong an experimental infection at the site of a delayed-type hypersensitivity reaction for as long as the sensitising agent (1-chloro-2,4-dinitrobenzene) was applied. Nevertheless, the lesions remained localised; furthermore, the experiment was carried no further than three weeks.

Histopathology of Experimental Infection

Pathological changes seen in experimental infections are similar to those seen in natural infections. *D.congolensis* invades the epidermis and the hair follicle sheath (Bida and Dennis 1977). In contrast, the dermis usually remains free of infection (Roberts 1965a).

With experimentally-produced lesions, the time course of infection may be studied. A comprehensive account of experimental infection of bovine skin is given by Oduye (1976b). Four hours after clipping, de-fatting and application of *D.congolensis*, dermal oedema and scattered PMN were observed in the area. After 24 hours a dense accumulation of PMN had separated the epidermis from the underlying dermis. Simultaneously, a new epidermis was forming underneath by proliferation and linking up of the follicular sheath epidermis. By 36 hours, the new epidermis was complete and demonstrated acanthosis. A layer of necrotic PMN had completely separated the old and new epidermis after 48 hours and by this time, the dermal cellular exudate had become predominantly lymphocytic. Most of the old epidermis, along with cell debris, was sloughed off as a scab 60 to 72 hours post-infection. Numerous macrophages had collected in the dermis by 96 hours and the dermal infiltrate remained a mononuclear one. Meanwhile, the new epidermis was still increasing in thickness up until 168 hours after which the thickness remained static. Occasionally, the new epidermis became infected and the whole process started again. From

his studies of experimental infection, Oduye (1976b) concluded that most reported differences in histopathology of cases of natural infection were due to the different stages of infection of the animals. In summary, PMN dominate the early lesion, whilst lymphocytes predominate later. The PMN are capable of phagocytosing *D.congolensis cocci* (Roberts 1966b) and thus prevent at least some of them from developing into filaments which are presumably too large to be ingested. The role of the lymphocytes has not been elucidated.

Other authors have noted the new epidermis in experimental infections to be hyperkeratotic, parakeratotic and acanthotic (Pulliam, Kelley and Coles 1967, Abu-Samra *et al* 1976a, Bida and Dennis 1977).

Since an experimental infection shares essentially the same characteristics with a natural one but for the chronicity and generalised distribution of the lesions, it follows that in the field, there must exist predisposing factors to a chronic as opposed to an acute, transient infection.

FACTORS PREDISPOSING TO A CHRONIC INFECTION

Many different factors have been ascribed to either a central or peripheral role in the aetiology of dermatophilosis. These can be divided into factors which cause mechanical damage to the skin and factors which suppress host immune mechanisms or general condition.

In the field, the intact skin surface is frequently breached, especially in pastures containing thorny bushes and where there is a high arthropod challenge. Zlotnik (1955) noted that most lesions on cattle were on the back where constant scratches arose from low branches and thorns; whereas cattle grazed on open pasture did not develop the disease. Hadrill (1987) reported an increased incidence of

dermatophilosis in cattle grazed on pastures with abundant acacia bushes; however, this is also the favoured environment of *Amblyomma variegatum* ticks (Kaiser 1987). Other factors which may cause damage to the skin and facilitate infection are the activities of the ox-pecker bird (Oduye 1976a), mange mites (Stewart 1972b) and nicks caused when shearing sheep (Roberts 1963d).

Dermatophilosis is predominantly a disease of the rainy season (Plowright 1956). Yet, it is unclear whether heavy rain has a direct effect of damaging the skin surface, or whether the association is an indirect one; whereby it facilitates release and transfer of motile cocci, or mediates increases in the arthropod populations which then cause the damage (Davis and Philpott 1980).

Burridge (1985) found the disease only on those Caribbean islands where *Amblyomma variegatum* ticks were present. Plowright (1956) noted that the areas of the body affected with lesions commonly correlated with the bite predilection sites of *Amblyomma variegatum*, namely the groin, scrotum, udder and axilla. In Antigua, *A.variegatum* infestation of cattle is common and yet the distribution of lesions does not correlate with tick attachment sites but are found mostly along the back which is subjected to a heavy challenge from biting flies, particularly *Haematobia* species (Hadrill 1987). Nevertheless, the instigation of a fly control programme, using pyrethroid insecticides, failed to diminish the incidence or alter the distribution of lesions (*Ibid.*). Stewart (1972b) also incriminated biting flies in contributing to the development of the disease.

Oduye (1976a) emphasised that a multitude of factors were involved in the aetiology of the disease. In his study of dermatophilosis in Nigeria, he implicated ticks, flies, soaking of the limbs through water-logging of pastures followed by trauma via thorny vegetation and *Demodex bovis*

infestation. In his opinion, the activity of biting flies is the predominant factor in the development of the generalised form of the disease, where the whole body is affected. This is due to the often disrupted feeding activities of the flies, whereby a large part of the body is bitten through constant alighting, disturbance and re-alighting of the flies.

The first requirement for infection is a breach in the integrity of the skin barrier but this is insufficient to produce a chronic lesion, as evidenced by the limitations of experimental infection (Davis and Philpott 1980). Therefore, it is likely that a number of factors, other than simple mechanical disruption, are involved in chronic cases.

Ticks and biting flies may have a dual role in facilitating the development of generalised dermatophilosis causing both mechanical damage and immunosuppression. A state of delayed hypersensitivity is known to develop at the site of arthropod bites (Reviewed by Benjamini and Feingold 1970) and Davis and Philpott (1980) proposed that this may suppress the normal, local host immune response. Lloyd (1984) suggested that a general state of immune deficiency may also contribute to the chronicity of the disease. Some evidence exists suggesting that tick infestation may suppress overall lymphocyte responsiveness (Wikel and Osburn 1982). The immune suppression might also arise through concurrent infections and the occurrence of dermatophilosis has been associated with that of trypanosomiasis, lumpy skin disease, orf and rinderpest (Plowright 1956, Stewart 1972b, Munz 1976). One electron-micrographic study of *D.congolensis* scabs, from natural cases of dermatophilosis in Nigeria, revealed the presence of pox virions in 77 per cent of the lesions and the authors suggested that a synergistic relationship may exist between the two pathogens (Isitor, Kazeem, Njoku, Adegboye and

Dellmann 1988).

Malnutrition has also been implicated in predisposing to dermatophilosis infection (Davis 1984). However, Oduye (1976a) was of the view that well-nourished animals are equally at risk, whereas, animals with the disease are often in poor condition because of impairment of mobility, due to leg lesions, and therefore of ability to feed. Malnutrition might cause a change in the resident skin flora, which could then allow opportunist pathogens, such as *D.congolensis*, to invade the skin (Jenkinson 1976).

RESISTANCE AND NON-SPECIFIC IMMUNE MECHANISMS

Prior infection with *D.congolensis* does not confer a complete immunity on the animal (Mémery and Thiéry 1960). However, accelerated healing of lesions following a previous infection has been reported (Roberts 1966a). An exception to this view of lack of immunity was the work of Abu-Samra *et al* (1976a). They reported that a range of animals tested did not develop lesions when scarified and re-inoculated with the organism 21 days after an initial experimental infection. From this, they proposed that experimental infection conferred a degree of transitory resistance.

In natural infection, however, animals are equally or more susceptible to re-infection. In their studies of bovine infection in the field, Mémery and Thiéry (1960), noted that the same animals in a herd developed dermatophilosis from one year to the next. During the dry season these animals had latent infections and when the wet season arrived, they relapsed, developing more serious lesions than previously. In the authors' opinion, this lowered resistance was due to the latent infection contributing to a poor, general state of health.

Certain breeds of animals are known to possess a degree of natural

resistance to dermatophilosis. In West Africa, the N'Dama and Muturu breeds of cattle appear to have a high degree of resistance to the disease and under normal conditions only develop sub-acute infections. In contrast, Zebu and all European breeds are highly susceptible to the chronic, generalised syndrome (Amakiri 1977, Marchot and Leroy 1987). In East Africa, the Dinka breed is also highly resistant (Leroy and Marchot 1987). This innate resistance may be inherited, since a study of natural infection in Sudan revealed that the degree of resistance of cross-breds decreased with decreasing amounts of Dinka and increasing amounts of exotic breed blood (*Ibid.*). The mode of inheritance is, however, unclear. Moreover, Lloyd (1976) found the offspring of N'Dama crosses did not possess any resistance.

Amakiri (1977) suggested the greater degree of resistance of the N'Dama as compared with the White Fulani, might be due to the higher percentage of lymphocytes and PMN which are present in their skin. In addition, variation in the normal skin bacterial flora between breeds might help to explain differences in susceptibility. Nwufoh and Amakiri (1981) observed that N'Dama cattle possessed a skin flora dominanted by *Bacillus* species whereas in susceptible breeds, such as Friesian and White Fulani, Staphylococci predominated. It is possible that the *Bacillus* species present on the skin produce substances which are inhibitory to *D.congolensis* growth because organisms in this genus are known antibiotic producers. In support of this view, Kingali (1987) demonstrated that *Bacillus spp.*, isolated from the skin of clinically normal sheep, produced substances capable of inhibiting *in vitro D.congolensis* growth.

Variation in susceptibility is also seen in sheep; Roberts (1963d), for example, found that Merino sheep were far less susceptible to experimental infection than coarse-wool breeds. The Merinos have a higher

density of sebaceous glands in their skin and they may therefore be more likely to maintain an intact sebaceous film. The layer of sebum is one of the three main skin barriers, the others being the hair or wool and the *stratum corneum* (Roberts 1967). Roberts (1963d) thought that the thickness of the *stratum corneum*, which varies with body site, also influenced the probability of infection. He found areas of the sheep with a thicker *s.corneum* were less likely to possess lesions. In contrast, Amakiri (1973) discovered that there was no correlation between lesion sites and thickness of the *stratum corneum* in N'Dama, Muturu and Fulani cattle in Nigeria. Likewise, Lloyd and Jenkinson (1980) found there was no relationship between the number of cell and lipid layers in the *stratum corneum* and the level of infection in cattle.

The efficiency of PMN in phagocytozing and destroying invading *D.congolensis* cocci may vary between different species. Roberts (1966b) observed that rabbits possessed a degree of natural resistance to experimental infection not shared by species such as sheep and guinea-pigs. He attributed this to a greater inhibition of growth of ingested *D.congolensis* cocci by rabbit PMN. In contrast to the findings of Roberts, Abu-Samra (1976a) observed that rabbits were just as susceptible to experimental infection as sheep and goats were.

Roberts was of the view that the response of PMN to the presence of *D.congolensis* is the most important host defence mechanism against the disease. Certainly, rabbits depleted of their PMN by treatment with nitrogen mustard required a lower dose of cocci to produce a confluent lesion than did normal rabbits (Roberts 1965b). However, it is impossible to relate this work to the number of cocci which naturally challenge the host in the field. Furthermore, the nitrogen mustard may inhibit the host reaction in ways other than the effect on PMN numbers (Turk 1975).

HUMORAL IMMUNE RESPONSE

Infection with *Dermatophilus congolensis*, either natural or experimental, is followed by the appearance of specific antibodies in the host serum. However, the antibody is not protective against re-infection. Several workers have demonstrated the presence of high titres of antibodies to *D.congolensis* after an experimental infection and yet shown that the animals are just as susceptible to re-infection (Merkal, Richard, Thurston and Ness 1972, Richard, Thurston and Pier 1976, Lloyd and Jenkinson 1980). Furthermore, a study of sixty naturally infected cattle revealed that there was no correlation between the severity of infection and the antibody titre (Oduye 1974).

Makinde and Ezeh (1981) described accelerated healing of lesions following a secondary experimental infection of Zebu cattle. Since this occurred in conjunction with an anamnestic response, the authors suggested that the high antibody titres might be associated with the accelerated healing. This idea is not supported by the work of Roberts (1966a) who demonstrated that animals given hyper-immune antiserum to *D.congolensis*, prior to infection, did not show accelerated healing, unlike those animals from which the serum came.

Animals may possess "natural" agglutinins which react in *in vitro* tests for *D.congolensis* antibodies. These non-specific antibodies are present only at low levels and are therefore easily distinguished from those specific antibodies produced in response to infection (Lloyd 1981). High antibody titres to *D.congolensis* can sometimes be found in cattle which apparently have never shown symptoms of dermatophilosis. Lloyd (1981) cites this as evidence of sub-clinical infection.

The first antibodies to appear following experimental infection are haemagglutinatins. These are initially detected between five days

(rabbits) and two weeks (cattle) after infection and occur with increased titres following re-infection (Richard *et al* 1976). Precipitating antibodies, on the other hand, are not produced after a single infection but are present after re-infection and also in natural chronic cases (*Ibid.*).

The role of antibodies in the host response to infection is uncertain. Daily treatment of rabbits with the cell replication inhibitor, methotrexate, abolished the normal precipitin response to infection and led to lower titres of haemagglutinating antibodies. Despite the inhibition of the humoral response, the treated animals followed the same course of infection as untreated controls (Merkal *et al* 1972).

Much of the work on the humoral response to *D.congolensis* has been carried out as part of the search for a feasible vaccine against dermatophilosis. Roberts (1966b) reported a sixteen-fold increase in the infective dose required following a vaccination schedule using inactivated *D.congolensis* cocci. The response was restricted to sheep and guinea-pigs whose skin had been scarified at the site of infection and there was no effect on rabbits or on infection at de-waxed sites. The partial protection seen in sheep and guinea-pigs was attributed to enhanced phagocytosis by PMN, brought about by the high antibody titres induced by the vaccination. The suggestion is feasible since binding of immunoglobulin (Ig) G antibody to bacteria activates complement, resulting in the release of chemoattractants for PMN. Thus, large numbers of these cells would be brought into the area, bind to, and ingest the bacteria (Roitt 1984). In contrast, Bida and Kelley (1976), although able to induce serum antibodies to *D.congolensis* following hyperimmunisation with several different antigen preparations, found that none gave protection against experimental infection.

Most studies of the humoral immune response to *D.congolensis* have

concentrated on serum antibodies. Lloyd (1984) suggested that local humoral immune mechanisms may be important. A comparison of immunoglobulin classes in the serum with those isolated from the skin itself showed that the two populations were not identical following intra-dermal vaccination with live *D.congolensis*. Although specific IgG titres in the skin and serum both increased after vaccination, specific IgM titres only increased in the skin and a complex temporal pattern of changing IgA titres was seen which was difficult to interpret (Lloyd and Jenkinson 1981, Lloyd and Jenkinson 1987). The function of the immunoglobulins is unknown; the serum antibodies were able to inhibit the motility of *D.congolensis* cocci, although they were not able to induce complement-mediated lysis (Jenkinson *pers.comm.*). Sutherland, Ellis, Robertson and Gregory (1987) also demonstrated anti-*D.congolensis* IgA in serum and skin washings following experimental infection of sheep. The increased IgA titres in the skin washings occurred later than those in the serum suggesting local production or selective transport. The same group of workers demonstrated IgA-bearing plasma cells in the cellular infiltrate at infection sites, the appearance of which preceeded the resolution of the lesion (Ellis *et al* 1987). However, on re-infection, the time of resolution and the type of cellular infiltrate varied markedly between individual animals and between different groups which had either undergone single or multiple biopsies during the period studied.

Thus, numerous studies of the humoral response to *D.congolensis* have identified specific antibody production, yet the protective role of these, if any, remains obscure.

CELL-MEDIATED IMMUNE RESPONSE

A delayed-type hypersensitivity reaction develops in response to invasion of the skin by *Dermatophilus congolensis* (Roberts 1966a). Lloyd (1984) suggested that, although the response does not seem to be protective, it may be related to the accelerated healing seen in previously exposed animals. He also proposed that the natural resistance of certain breeds of cattle is partly due to the cell-mediated immune response to infection which these animals mount.

Most studies of the cell-mediated response to *D.congolensis* have centered on experimental infection. Roberts (1966a) assessed the response of various animals to infection by measuring the diameter of erythema induced by intra-dermal injection of *D.congolensis* antigen, taking this as an index of hypersensitivity. He reported delayed-type hypersensitivity (DTH) responses to primary and secondary experimental infections in sheep, rabbits and guinea-pigs. An immediate hypersensitivity reaction also occurred but only after more than one infection. The DTH response appeared to be of the tuberculin type and would therefore have been mediated by mononuclear cells. It first appeared four days after commencement of a daily infection schedule which was also the time at which the lesions began to resolve. Roberts (*Ibid.*) suggested that acceleration of the cellular response caused the cessation of invasion of sheep skin by *D.congolensis*. It is interesting to note that chronically infected sheep developed a DTH response to experimental infection of unaffected areas. Accelerated healing of these new lesions occurred despite the persistent natural infection (*Ibid.*).

Merkal *et al* (1972) found that the lesions on experimentally-infected rabbits developed and resolved in similar fashion whether or not the animal had been treated with methotrexate. Methotrexate is an inhibitor

of cell division which suppresses both DTH and immunoglobulin production. The authors concluded that recovery from infection depended on non-specific inflammatory responses which are not affected by methotrexate. However, the conclusion was reached without any attempt to determine whether a DTH response occurred or not. Furthermore, depending on the dose of methotrexate and the mode of antigen administration, the agent can inhibit antibody production, whilst not affecting DTH (Friedman, Buckler and Baron 1961). In contrast to the work of Merkal *et al*, administration of another immunosuppressive agent, cyclophosphamide, was found to greatly prolong the duration of experimentally-induced *D.congolensis* lesions in rats (Morrow *pers. comm.*).

Makinde and Wilkie (1979) investigated the cell-mediated response of rabbits to experimental infection with *D.congolensis*. Intra-dermal injection of *D.congolensis* antigen into previously infected animals, led to induration suggestive of a DTH response. They reported that lymphocytes isolated from experimentally-infected rabbits responded to *D.congolensis* antigen *in vitro*, as determined by a lymphocyte transformation test. However, the pattern of response was unusual in that a strong stimulation of lymphocytes on day ten was followed by no response by cells on day fifteen, then by another strong stimulation of day-twenty cells. Furthermore, the *in vitro* response did not correlate, in respect to time, with the *in vivo* one of skin tests. A futher index of cell-mediated immune responses was also examined. Culture supernatants of *D.congolensis* antigen-stimulated lymphocytes were assessed for the presence of macrophage migration inhibition factor (MMIF). Again, the authors obtained unusual results in that an indirect macrophage migration assay showed the supernatants to have stimulatory rather than inhibitory effects on migration. The result could be explained if the authors

measured the migration distances later than the conventional time of 24 hours after setting up the test because other factors in the supernatants may become active following a period of migration inhibition (Salvin 1974). Unfortunately, the authors did not state the time at which they assessed the result of the test.

Higgins (1983) demonstrated macrophage migration inhibition by cells from infected rats when stimulated by antigen in a direct test for MMIF. He found the onset of MMIF activity to occur three days after infection, with an increasing percentage of rats showing migration inhibition with time, up to a maximum of 100 per cent by day ten.

Research on the cell-mediated response during natural infection has been neglected. Abu-Samra, Imbabi and Mahgoub (1976b) noted that the histopathological changes seen in naturally infected Sudanese cattle were suggestive of a progressive disease of the tuberculin delayed hypersensitivity type. These particular changes were of severe dermal reactions characterised by lymphocyte, macrophage and giant cell infiltration. Abu-Samra (1980) pointed out that the reaction to experimental infection may be different to that in natural infections. He thought that the massive doses given to elicit an experimental infection were unrepresentative of the repeated small doses received through damaged skin barriers in the field. His proposal was that these repeated small doses give rise to a state of hypersensitivity with the release of mediators such as MMIF, chemotactic and lymphotoxic factors.

In the field, DTH may also develop to agents other than *D.congolensis*, such as biting-arthropods. These reactions may interfere with the normal hypersensitivity response to *D.congolensis*. In an attempt to mimic the effect of biting-arthropods, Davis and Philpott (1980) induced a DTH response to 1-chloro-2,4-dinitrobenzene (DNCB) and then inoculated the

same site with *D.congolensis*. They reported that the lesions induced were morphologically similar to those in natural infections and persisted for as long as the DNCB was applied. The authors proposed that a DTH reaction to an arthropod bite in the field may be followed by a period of immune suppression at that site due to feedback mechanisms operating. Thus, no immune response would develop if the site became invaded by *D.congolensis* and the infection would therefore progress to a chronic state. Davis (1984) later used the number of *D.congolensis* cocci recovered from infected guinea-pigs as an index of degree of infection. He found that animals which were sensitised to DNCB yielded higher numbers of cocci, and for longer periods, than DNCB-naive animals. In contrast, fewer cocci could be harvested from those guinea-pigs made tolerant to DNCB than those sensitised to it. This work suggests that *D.congolensis* can establish itself more easily where hypersensitivity reactions to other agents are present. Thus, it might be thought that where an animal is undergoing a DTH response to an unrelated agent, the cell-mediated response to *D.congolensis* at that site would be inhibited. Yet, Higgins (1983) found that rats sensitised to DNCB showed the same time course of macrophage migration inhibition activity as controls.

Clearly, the immune response to *Dermatophilus congolensis* requires further elucidation. In the field, concurrent immune responses to other agents add a futher dimension of complexity. The advantage of investigating the response to experimental infection is that factors of interest can be studied in isolation. Furthermore, the response to an experimental infection may be analagous to a successful response to natural infection because, in both, the infection is rapidly resolved.

DEFENCE MECHANISMS OF THE SKIN

NON-SPECIFIC DEFENCE MECHANISMS

The skin provides an effective barrier to the entry of most potential pathogens. Non-specific inhibitors of microbial growth and invasion include the integrity of the *stratum corneum*, a low surface pH, fungistatic free fatty acids in the sebum, desiccation and competition by the resident microbial flora (Reviewed by Rothman and Lorincz 1963). In addition, host factors such as transferrin and α_2-macroglobulin may hinder fungal spread in dermatophyte infections, by iron sequestration and keratinolytic-enzyme inhibition (King, Khan, Foye and Greenberg 1975 and Yu, Grappel and Blank 1972, respectively)

Active processes such as desquamation of the cornified cells and increased keratinisation following mechanical stimulation also play a role in preventing the establishment of pathogens (Rothman and Lorincz 1963). Genetic variability in the expression of these factors may contribute to the different susceptibilities of individuals to certain skin diseases.

THE SKIN IMMUNE SYSTEM

In recent years it has become clear that elements within the skin play an active role in immune protection. Streilein first proposed the existence of skin-associated lymphoid tissue (SALT) in 1978 and since then a growing body of evidence has accumulated in support of the concept that a part of the immune system is specialised to provide protection for the skin. Some T-cells may preferentially locate within the epidermis, which is also the site for antigen processing and presentation; interaction between primed lymphocytes and specific antigen may occur

within the skin, with regulation of the response at the site (Reviewed by Streilin 1985).

The concept of SALT has been criticised due to differences from the other tissue-associated lymphoid tissues, namely, those in the gut and bronchus. Bos, Zonneveld, Pranab, Das *et al* (1987) observed that B-cells were apparently absent from normal human skin and epidermal T-cells were found in very low numbers. In turn, the work of Bos and colleagues has been questioned, with the identification of "significant numbers" of T-cells, particularly of the T-helper type in normal human epidermis (Rowden, Davis, Luckett and Poulter 1988).

Antigen Presentation in the Skin

The most well-defined antigen presenting cell (APC) found in the skin is the Langerhans cell which is an epidermal Ia (class II)-expressing dendritic cell (Stingl, Katz, Shevach, Wolff-Schreiner and Green 1978). Langerhans cells are as efficient at processing and presenting antigen as monocytes and macrophages (Reviewed by Bagot, Heslan, Dubertret, Roujeau, Touraine and Levy 1985) and in common with these cells, secrete interleukin-1 (Sauder, Dinarello and Morhenn 1984). Langerhans cells are most often found in the suprabasal layers of the epidermis (Stingl, Tamaki and Katz 1980) but are mobile cells which circulate between blood and epidermis via the draining lymph node (Shimada and Katz 1988).

Kaplan, Nusrat, Witmer, Nath and Cohn (1987) followed the distribution of Langerhans cells during a DTH response to tuberculin in lepromatous leprosy patients. They noted increased epidermal cell proliferation following tuberculin injection, with the Langerhans cells seemingly caught up in the flow of epidermal cells, such that they were shed from the skin. The epidermis was eventually repopulated with Langerhans cells,

following migration up from the cellular infiltrate in the dermis.

Ia-postitive dendritic cells, of unknown function, also reside in the dermis and appear distinct from Langerhans cells in lacking the surface antigen T6 (Headington and MacDonald 1984).

Granstein (1985) identified another epidermal APC after ultra-violet (UV) light abrogation of Langerhans cell function. The UV-resistant APC induced antigen-specific immune suppression of a contact hypersensitivity response. Unlike the Langerhans cell, the APC was Ia-negative and activated T-suppressor cells.

Okamoto and Kripke (1987) demonstrated activation of the immune effector and of the immune suppressor pathways for contact hypersensitivity. APC (Langerhans cells) could be found in the draining lymph node following topical application of a contact sensitising agent, presumably after having migrated there from the epidermis. These cells were able to transfer antigen-specific contact hypersensitivity but in their absence, Ia-negative, Thy 1-positive cells induced antigen-specific suppression. At present, the Ia-negative, Thy 1-positive cell is thought to be distinct from the APC cell described by Granstein (Rowden *et al* 1988); it shares the same lineage as T-cells and may be an immature T-cell (Stingl, Gunter, Tschachler, Yamada *et al* 1987). Indeed, Streilin (1978) suggested the skin may be a site for T-cell maturation.

Role of Epidermal Cells in the Immune Response

In addition to the epidermal APC, the keratinocytes themselves may play a role in the development of immune responses. Keratinocytes secrete epidermal cell-derived thymocyte activating factor (ETAF) (Sauder, Carter, Katz and Oppenheim 1982) which is probably identical to interleukin-1 (Sauder 1985). Although epidermal cells constitutively

produce interleukin-1 (IL-1), synthesis is increased by stimuli such as lipopolysaccharide and cell damage (Luger, Stadler, Luger, Mathieson *et al* 1982). Large amounts of IL-1 have been isolated from normal *stratum corneum* but it is not known if this simply represents a means of IL-1 disposal via desquamation (Gahring, Buckley and Daynes 1985). Alternatively, the source of IL-1 could become important following damage, caused by wounding or infection, when IL-1 release into the surrounding skin may participate in inducing an inflammatory response (*Ibid.*).

IL-1 is a chemoattractant for both PMN (Sauder, Mounessa, Katz, Dinarello and Gallin 1984) and T-cells (Sauder, Monick and Hunninghake 1984). The release of IL-1 may therefore contribute to the leucocyte infiltrates, characteristic of many skin disorders, as well as causing the activation of the T-cells within them. The effect of IL-1 on epidermal and fibroblast growth will be discussed in chapter six.

In addition to IL-1, epidermal cells release interleukin 3-like factor (Luger, Wirth and Kock 1985) and natural killer cell activating factor (Luger, Uchida, Kock, Colot and Micksche 1985) which may contribute to mast cell and natural killer cell activity respectively during reactions in the skin. Lymphokines which are released by non-lymphoid cells have been termed cytokines (Luger, Kock, Danner, Colot and Micksche 1985). Another characteristic of many skin disorders, discussed in chapter seven, is the expression of class II (Ia) antigen on epidermal cells.

Cell-Mediated and Humoral Immunity

Dermatophyte infections typically elicit cell-mediated responses; humoral responses, where they occur, do not appear to be protective (Jones and Artis 1981). In support of this view, Calderon and Hay (1984)

were able to confer a degree of protection against *Trichophyton quinckeanum* in sub-lethally irradiated mice by transfer of sensitised T-cells but could not confer protection in normal mice by transfer of antibody-containing serum from the same donor. Moreover, patients with defects exclusively in humoral immunity seldom develop the chronic skin disease, mucocutaneous candidiasis whereas the disease is common in those with cell-mediated defects (Kirkpatrick and Sohnle 1981).

Lepper (1972) observed that the resolution of bovine *Trichophyton verrucosum* infections coincided with the appearance of a positive DTH skin test to specific fungal antigen. In contrast, chronic cases are often characterised by a lack of reactivity to dermatophyte antigens, assessed by either skin testing or lymphocyte transformation tests (Hanifin, Ray and Lobitz 1974).

Antibody-Dependent Cellular Cytotoxicity

Antibody-dependent cellular cytotoxicity is an important defence mechanism in some skin infections (Reviewed by Norris 1985). Target cells become coated with specific immunoglobulins and are then lysed by Fc receptor-bearing nonspecific effectors. The effector cells are macrophages, large granular lymphocytes, T-cells, eosinophils and PMN, with the last being the usual effector against bacteria. The lytic factor appears to be lymphotoxin released by T-cells and hydrolytic enzymes or toxic oxygen products released by macrophages and PMN.

Complement

Complement does not appear to play an important role in host defence against dermatophyte infections (Jones and Artis 1981). In contrast, complement components are important in limiting the downwards spread of *Candida spp.* skin infections (Ray and Wuepper 1978) and may arise following direct activation of the alternative complement pathway by *Candida spp.*, which in turn, elicits an inflammatory response (Ray and Wuepper 1976).

Role of Phagocytosis in the Response to Skin Infections

Leucocytes may be attracted to an infection site either by the mechanisms outlined above, (p33) or, for PMN, by chemotaxis in response to complement components arising via the alternative pathway (Roitt 1984). A variety of effector mechanisms then come into play. The phagocytic cells, macrophages and PMN are involved in the response to a number of skin infections and phagocytosis is the usual means of destroying bacteria (Roitt, Brostoff and Male 1985). Phagocytosis occurs following adherence of the cell to the target and is enhanced by complement or antibody opsonisation (Roitt 1894). However, phagocytosis is probably not an important defence mechanism where the pathogen is filamentous due to the discrepency in size between the phagocyte and the potential target (Jones and Artis 1981). Epidermal cells may also contribute to the phagocytic destruction of invading microorginisms; Csato, Bozoky, Hunyadi and Dobozy (1986) have demonstrated that isolated epidermal cells are capable of phagocytosing and killing *Candida albicans*.

Some skin pathogens such as *Mycobacterium leprae, M. tuberculosis* and *Leishmania spp.* have evolved means of resisting lysis once ingested (Roitt *et al* 1985). To be able to eliminate pathogens, macrophages must

first be attracted to the area and then activated; lymphokines, secreted by sensitised lymphocytes, play a major role in this process. It is not certain where the immune defect lies in chronic diseases such as lepromatous leprosy (Turk 1981). However, abnormal macrophage behaviour has been observed in lepromatous but not tuberculoid leprosy patients, such as depressed protein synthesis (Salgame, Birdi, Mahadevan and Antia 1981) and reduced ability to process *M. leprae* antigen following phagocytosis of the organism (Birdi, Salgame, Mahadevan and Antia 1981).

Thus, the skin plays an active role in defence against potential pathogens. Exposure to foreign components is followed by antigen processing and presentation and subsequent elicitation of a variety of immune effector cells at the site. The release of cytokines from epidermal cells and lymphokines from mononuclear cells allows interaction between the immune system and the skin. Regulation of the response is made possible by mechanisms such as activation of specific suppressor cells via APC other than Langerhans cells.

FIGURE 1.1 A natural case of generalised dermatophilosis from Antigua.

FIGURE 1.2 Close-up of the back of a naturally-infected cow showing extensive scabs.

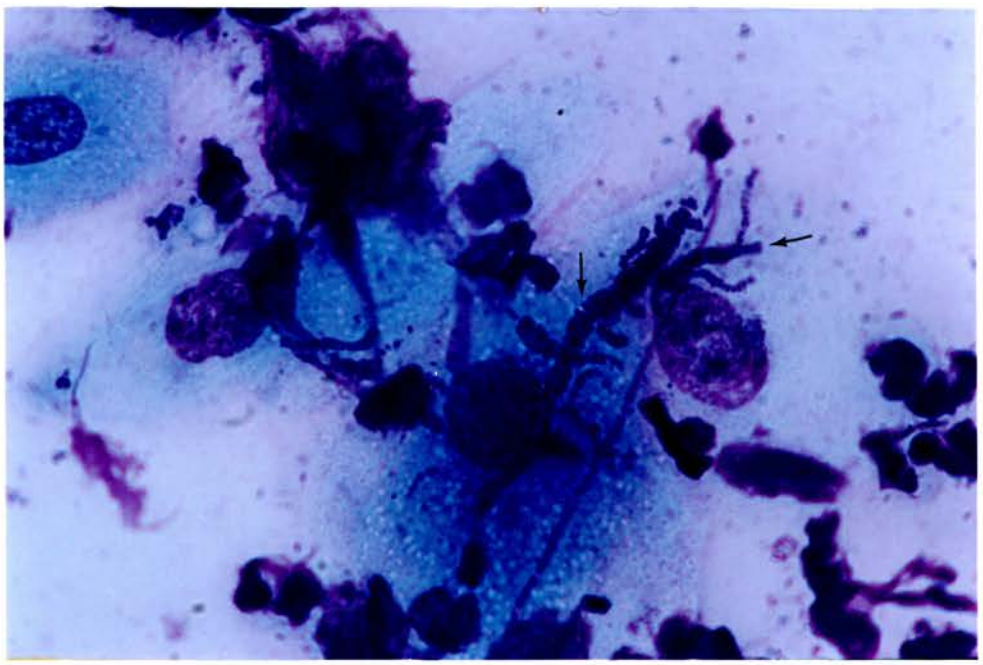
(Photographs courtesy of Dr.Morrow)

FIGURE 1.3 Giemsa-stained smear prepared from the underside of a scab taken from an animal with generalised dermatophilosis showing *Dermatophilus congolensis* filaments composed of multiple rows of cocci (arrows).

(x 1,060 magnification)

FIGURE 1.4 Experimentally-induced *Dermatophilus congolensis* infection of a rat. Note the localisation of the lesion to the original site of application of the organism.

(Photographs courtesy of Dr.Morrow)



CHAPTER TWO

RESPONSE OF MONONUCLEAR CELLS TO INFECTION WITH *DERMATOPHILUS CONGOLENSIS*

CONTENTS (cont.)

CONTENTS (cont)

INTRODUCTION

Several studies on antibody titres to *D. congolensis* following infection have been conducted. In contrast, little is known about the response of T-cells to infection with *D. congolensis*. An investigation was undertaken into whether rat mononuclear cells could be primed by *in vivo* exposure to *Dermatophilus congolensis*, or its components. The subsequent *in vitro* response of these cells to the organism was then examined.

The exact relationship between *in vitro* mononuclear cell (MC) responses and the normal host immune response is not clear. Mills (1966) demonstrated a good correlation between lymphocyte transformation *in vitro* and the state of delayed hypersensitivity *in vivo*. In addition, Myrvang, Godal, Ridley, Froland and Song (1973) found that the response to *Mycobacterium leprae* in the lymphocyte transformation test correlated with the disease state. Lymphocytes from tuberculoid leprosy patients responded well, whereas, those from lepromatous leprosy patients did not. However, there is some doubt that the reaction to *M. leprae* in the lymphocyte transformation test is to the antigens which are associated with host resistance (Turk 1981). Nevertheless, the lymphocyte transformation test is widely used as an indicator of immune status (Maluish and Strong 1986).

Before the mononuclear cell response to *D. congolensis* could be assessed isolation and culture protocols had to be devised which would yield cells amenable to *in vitro* stimulation. Furthermore, it was necessary to find an effective route of antigen administration which would ensure sensitisation. Thus, a known antigen, purified protein derivative of *Mycobacterium tuberculosis* (PPD), was chosen for this purpose. PPD was administered by two different routes and the subsequent *in vitro* response

of MC to a range of PPD concentrations assessed.

The effect of *D.congolensis* antigen on MC, derived from rats sensitised to the antigen, was investigated using the same protocol as that which had proved successful for PPD. In addition, MC were isolated from *D.congolensis*-infected rats and the responses to *D.congolensis* antigen and to intact cocci were assessed with a ^{3}H-thymidine incorporation, lymphocyte transformation test.

MATERIALS AND METHODS

ANIMALS

Inbred male Wistar rats, approximately three to five months-old (Bantin and Kingman), were used as the source of blood and spleen for mononuclear cell isolation. Prior to isolation, the rats were sensitised to antigen or infected with *Dermatophilus congolensis* where appropriate.

CELL CULTURE TECHNIQUES AND MATERIALS

Aseptic technique was used in the preparation of tissues for culture and throughout all the culture work reported in this and later chapters. The water used for preparation of medium and of other solutions was of tissue culture grade (Appendix 1). Materials for culture-use were sterilised by the appropriate method (Appendix 1). Prior to use, all foetal calf serum (FCS) was heated to 56°C for 30 minutes to inactivate complement and stored at -20°C. Glassware was washed to tissue culture standard (Appendix 1).

MONONUCLEAR CELL ISOLATION

Initially, MC were isolated from rat blood (Appendix 1) thereafter, the spleen was used as the source of MC. Prior to spleen removal, the rat was killed by cervical dislocation and placed with its left side uppermost. The flank was swabbed with 70 per cent alcohol and the skin cut away to expose the peritoneal wall. The peritoneal cavity was opened with fresh sterile instruments and the spleen removed into RPMI-1640 incomplete medium containing 2 units ml^{-1} heparin, 2 percent FCS, 200 units ml^{-1} penicillin and 200 μg ml^{-1} streptomycin (Appendix 1). The spleen was rinsed with incomplete medium and any attached fat removed.

Using curved scissors and forceps, the spleen was finely chopped in fresh incomplete medium and the resulting suspension gently mixed, using a wide-bore pipette to further disaggregate the tissue. The suspension was then passed through a 230 μm stainless steel mesh[1], using more medium and a pestle to grind up remaining solid tissue. To achieve a single cell suspension, the spleen mixture was passed through a 94 μm mesh and then rinsed through with medium.

The final suspension (volume about 36 ml) was divided into two halves and each carefully layered onto 8 ml pre-chilled Ficoll-paque (Ficoll 400 plus sodium diatrizoate, total density 1.077 g ml^{-1}, Pharmacia). A range of centrifugal forces were tested to determine the optimum for mononuclear cell separation (Appendix 1). The Ficoll-gradients were centrifuged with the optimum force at the interface of 480g, for 30 minutes at 15°C. The cell-free supernatant from the gradient was discarded. The band below this, which was rich in lymphocytes, was collected, as was some of the Ficoll layer underneath, which contained monocytes and lymphocytes. The pellet at the bottom of the gradient consisted of the majority of erythrocytes and granulocytes, together with some MC and damaged cells; this was discarded. The harvested MC were washed in 20 ml phosphate-buffered saline (PBS) with centrifugation at 300g for 5 minutes, 15°C, to remove platelets and Ficoll-paque. This wash was repeated and the cell pellet finally resuspended in complete RPMI-1640 medium (Appendix 1). Aliquots were taken at the various stages of separation for cell counts and cytospins. The viability of the end product was also determined. Whilst these were being carried out the cells were kept at 4°C.

[1]Bellco cellector

CELL AND VIABILITY COUNTS

The determination of leucocyte concentration was performed using an electronic particle counter (Coulter counter[1]). The Coulter counter was first calibrated for rat leucocytes to determine the appropriate settings for future counts. Samples to be counted were well mixed and a 20 µl aliquot was diluted 1/500 with Isoton II[1] using an automatic DD10 diluter/dispenser[1]. Erythrocytes were lysed by the addition of 6 drops of Zapoglobin[1] per 40 ml of the diluted sample. Duplicate counts were then performed on each sample.

The accuracy of the Coulter counts was checked by comparing the values with those derived from haemocytometer counts of the same samples. These were carried out with an improved Neubauer haemocytometer, using the method described by Mishell, Shiigi, Henry and Chan (1980).

For viability counts, aliquots of cell suspensions were diluted by a factor of ten with a 0.2 per cent solution of trypan blue (Sigma) made up in PBS and stored at 4°C. The suspension was gently mixed and left to stand at room temperature for five minutes to allow uptake of the dye. The two chambers of a Neubauer haemocytometer were filled with samples from the suspension and at least 200 cells were counted in each. The numbers of dye-excluding (viable) and dye-absorbing (non-viable) cells were recorded and the percentage of viable cells calculated.

CYTOCENTRIFUGE PREPARATIONS

Giemsa-stained preparations (cytospins) of cells were made by dropping 50-100 µl sample into the well of a cytocentrifuge (Cytospin II, Shandon). The cells in each well were spun onto clean glass slides by

[1]Coulter Electronics

centrifugation at 110g for 5 minutes. The slides were air-dried and fixed in methanol for 1-2 minutes, then stained with a five per cent solution of Giemsa's stain for 40 minutes (Appendix 1).

SPLEEN MONONUCLEAR CELL CULTURE

A range of culture conditions was investigated to determine those suitable for rat spleen mononuclear cells (SMC). FCS (Myoclone Plus, Gibco) was included in the medium at a level of eight per cent. This value was chosen as a compromise between better viability, but greater background stimulation with higher levels of FCS (ten per cent and above) and poorer viability, but lesser background stimulation with lower levels of FCS (five per cent and below). The background stimulation was inferred from the blast index of cells cultured in medium alone. The complete culture medium, routinely used for rat mononuclear cell culture was RPMI-1640 containing 25 mM Hepes, 8 per cent FCS, $5x10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine , 100 units ml^{-1} sodium benzyl-penicillin and 100 μg ml^{-1} streptomycin sulphate (Appendix 1).

The cells were cultured in either flat-bottomed 48-well plates (Costar) or flat-bottomed 96-well plates (Nunclon). The 1 cm^2 wells of 48-well plates were seeded with cells at an initial density of $1x10^6$ cells/well, in a total of 1 ml. For cultures in 96-well plates, the initial cell density was $2x10^5$ cells/well (0.38 cm^2) in a total of 200 μl ($1x10^6$ ml^{-1}): this was found to give far better viability than the $1x10^5$ cells/well used in earlier experiments where cytospins of day five cultures often demonstrated a low number of intact cells in the absence of stimulant.

The outside rows of wells were filled with sterile PBS, rather than used for cell cultures due to the susceptibility to evaporation. When

setting up cultures in multi-well plates, the cells were always added after the other constituents, to minimise the exposure time to unfavourable conditions. The plates were incubated at 37°C in a 5 per cent carbon dioxide-95 per cent air, humidified atmosphere.

The cultures were replenished with fresh medium on day three of culture. Half of the spent culture medium of each well was carefully drawn off, so as not to disturb the cells which were settled at the bottom. This was then replaced with fresh medium, containing antigen or mitogen where appropriate, such that the concentration of stimulant in the culture was maintained.

RESPONSE OF SPLEEN MONONUCLEAR CELLS TO THE ANTIGEN PPD

Wistar rats were sensitised to purified protein derivative of *Mycobacterium tuberculosis* (PPD)[1] by either intra-dermal or subcutaneous injection of 0.1 ml of 150 μg PPD in Freund's incomplete adjuvant (Appendix 1). Control animals were given a dose, at the same site, of 0.1 ml incomplete Freund's, emulsified with an equal volume of sterile PBS.

To determine whether the rats were sensitised to PPD, skin tests were carried out. Two test and two control animals were given 50 μg PPD in 0.05 ml sterile PBS intradermally in the ear, ten days after the initial sensitising-injection, after swabbing with alcohol and marking the site. The skin thickness at the sites was measured prior to injection and after 24 and 48 hours, with a micrometer (Dial calipers).

SMC were isolated from the rats ten days after sensitisation. Cells from both sensitised and control rats were cultured in 48-well plates, in

[1]The PPD was donated by the Central Veterinary Laboratory, Weybridge.

complete medium either alone, or with PPD at 5, 10 or 20 μg ml^{-1}, or with concanavlin A (Con A) as a positive control. Con A was included at the previously determined optimum concentration of 2 μg ml^{-1}. Both PPD and Con A solutions were made up in complete medium. On days three, five and seven of culture, four samples from replicate wells were taken for preparation of cytospins and two samples taken from further replicate-wells for Coulter counts and viability counts. Blast indices were calculated from the differential counts from the cytospins. The blast index is a measure of the proportion of stimulated cells; it is defined as the percentage of lymphocytes which are blastoid ie. are typically large, with a dark blue cytoplasm, paler nucleus and small regular vacuoles when observed on a Giemsa-stained cytospin. In contrast, unstimulated lymphocytes are small with a dark blue nucleus and a rim of pale blue cytoplasm (Ling and Kay 1975).

THE RESPONSE OF SPLEEN MONONUCLEAR CELLS TO *DERMATOPHILUS CONGOLENSIS*

DERMATOPHILUS CONGOLENSIS ISOLATE

The isolate of *Dermatophilus congolensis* used in these studies (A5N), originated from a clinical case of dermatophilosis in a sheep from the Pentland Hills, Scotland. A primary culture, which had been checked for purity, was stored in Columbia broth (Gibco) with 15 per cent glycerol (BDH) at -20°C.

Secondary cultures were grown up from aliquots of primary culture by the method described below. The secondary cultures were stored at -20°C in 100 μl volumes in 15 per cent glycerol and thawed when required. Thus, the *D.congolensis* isolate was subjected to no more than two cycles of freeze-thawing and two subcultures.

DERMATOPHILUS CONGOLENSIS CULTURE

A 100 µl aliquot of stock *D.congolensis* was thawed and divided between two blood agar (B/A) plates (Appendix 1). These were incubated at 37°C for 48 hours in 5 per cent carbon dioxide-95 per cent air and then for a further 24 hours at 37°C in air. Bottles of 15 ml brain heart infusion-neutralised soya peptone (BHI/NSP) broth (Appendix 1) were seeded with a loopful of *D.congolensis*, taken from the last confluent streak on the B/A plate. After 72 hours at 37°C, 0.5 ml aliquots of broth culture were transferred to B/A plates which were then incubated for 48 hours at 37°C. *D.congolensis* cocci were harvested from the plates by the addition of 1.5 ml of sterile PBS, followed by gentle rubbing over the surface of the cultures with a bent pasteur pipette.

RESPONSE TO *D.CONGOLENSIS* ANTIGEN BY SMC FROM ANTIGEN-SENSITISED RATS

Preparation of *D.congolensis* Antigen

Crude antigen preparations were made from the two different growth forms of *Dermatophilus congolensis*, the filamentous form which is found in liquid culture and the motile coccoid form which can be harvested from solid media culture. Both types of antigen were prepared by ultrasonic disintegration of washed cultures, after which the soluble fraction was collected (Appendix 1). The protein content of the antigen preparation was determined using the BCA protein assay kit (Pierce).

Administration of *D.congolensis* Antigen and Skin Testing

Two protocols were used in an attempt to induce sensitisisation of rats to *D.congolensis*. In the first, one 150 µg dose of coccoid-form antigen, dissolved in PBS, was emulsified in an equal volume of incomplete Freund's adjuvant and injected intra-dermally into the rat in a total

volume of 0.1 ml. The second protocol involved three 150 μg doses of filamentous-form antigen preparation administered by the same method and route at ten-day intervals. Rats to be used as the positive control were given either a single dose (first experiment), or a triple dose (second experiment) of PPD at the same time(s) and by the same method as for the *D.congolensis* antigen. Negative control animals were given doses of 0.1 ml incomplete Freund's, emulsified with an equal volume of sterile PBS, again at the same site(s) and time(s).

Skin tests were performed on replicate animals on the day of SMC isolation. The method was that used for the PPD-sensitised rats (p.48). The amount of *D.congolensis* antigen injected into the test ear was 50 μg.

In Vitro Response to *D.congolensis* Antigen by SMC from Antigen-Sensitised Rats

SMC were isolated from rats ten days after the (final) dose of antigen. The cells were cultured in 48-well plates in complete RPMI medium, with a range of concentrations of *D.congolensis* antigen. The antigen (coccoid or filamentous) was dissolved in complete medium, filter sterilised (low protein absorption filter), diluted and added to the wells. PPD was added to appropriate wells at a concentration of 20 μg ml^{-1}, other wells received medium only; the total volume in each well was 1 ml. For each treatment, four replicate wells were set up for cytospins and two for cell counts, performed on days three, five and seven of culture.

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RESPONSE TO *D.CONGOLENSIS* ANTIGEN AND COCCI BY SMC FROM INFECTED RATS

Infection of Rats with *D.congolensis*

D.congolensis cocci were harvested in PBS from 48 hour-growth B/A-plate cultures (p.50). The suspension was incubated for 1 hour at 37°C to allow the filaments to settle out. The supernatant, containing motile cocci, was collected. Aliquots were taken for viable counts (Appendix 1) and purity checks by means of a Gram stain (Appendix 1) and streaking out on B/A plates which were then incubated at 37°C.

The flank of a rat was shaved and the area rubbed with ether to remove surface sebum. When the ether had evaporated, the site was lightly scarified with ten strokes in two directions using a number 40 blade from hair clippers (Oster); at no time did this process draw blood. Once scarified, 25 µl of the *D.congolensis* suspension, containing 2-12x10^6 cocci, was applied and spread evenly over the site. Second and third infections were induced, at different sites, at ten-day intervals. The rat was checked daily to monitor the course of infection. At the same time that the rats were infected with *D.congolensis*, positive control rats were sensitised to PPD by intradermal injection of 150µg PPD in Freund's incomplete adjuvant (p.48). Negative control rats were scarified in the same way and at the same times as the test rat but without the application of *D.congolensis*.

^{3}H-Thymidine Incorporation Lymphocyte Transformation Test

SMC were cultured, at an initial density of 1x10^6 cells ml^{-1}, in 200 µl volumes of complete medium, with or without *D.congolensis* antigen/cocci, in 96-well flat-bottomed plates. Eight replicate cultures were set up for each treatment. On day three of culture, half of the spent medium was

removed and replaced with fresh medium containing antigen/cocci where appropriate to maintain the concentration. On day five the cultures were pulsed with ^{3}H-thymidine. The dose and labelling period used in the assays were previously determined (see below).

Stock ^{3}H-thymidine (specific activity 5 Ci mmol^{-1}, concentration 1 mCi ml^{-1}, Amersham) was diluted by a factor of 100 with complete medium to 10 µCi ml^{-1}. Each well received 50 µl of the diluted label which contained 0.5 µCi of ^{3}H-thymidine, giving a final concentration of 2 µCi ml^{-1}. Eight extra cultures were included; these received no label and gave a measure of the background activity. The labelled cultures were incubated, under normal culture conditions, for the pre-determined time of four hours.

At the end of the incubation period, the cells were harvested onto glass fibre filter paper (Whatman) and washed, using a Dynatech Multimash 2,000 automated cell harvester[1]. The filter papers were dried in a hot-air oven; then each disc, which corresponded to one well of the plate, was transferred to a scintillation vial (Packard). To each vial 1 ml of scintillation fluid was added (Optiscint Hi-safe, LKB). The amount of tritium on the discs was determined with a Packard Tri-carb 2000 CA liquid scintillation analyser[1]. Each vial was counted for five minutes.

Results were expressed as counts per minute (CPM) which is more appropriate than disintegrations per minute (DPM) for this type of isotope counting. This is because DPM is a value derived from CPM, which takes into account the effects of chemical and light quenching. However, most of the quenching in this system is physical, being caused by the filter, yet this would not be included in the machine's calculation of

[1]Donated by the Wellcome Trust

DPM. This is true wherever the activity remains on the filter, as it does here, rather than eluting into the solution.

Determination of Labelling Conditions for Lymphocyte Transformation Test

SMC, isolated ten days after the third infection was induced, were incubated in the presence or absence of 6.7×10^6 ml^{-1} of *D.congolensis* cocci. This concentration was sufficient to cause significant stimulation of the cells (see p.70). Eight replicate cultures were labelled with a range of concentrations of ^{3}H-thymidine and incubated for a range of times. All cultures were harvested at the same time, on day five of culture.

In Vitro Response by SMC from Infected Rats to *D.congolensis* Antigen

SMC were isolated from rats ten days after the third infection. Cells from control rats were isolated at the same time, ten days after the the last PPD-sensitisation dose, or ten days after the last scarification (p.52). The cells were cultured in 96-well plates, as previously described (p.52). Filamentous antigen derived from *D.congolensis* was tested at ten-fold dilutions, giving final culture concentrations of 0.1 μg ml^{-1} to 10 mg ml^{-1}. Control cultures received either PPD at 20 μg ml^{-1}, or no additions to the medium. The response was assessed by the ^{3}H-thymidine incorporation lymphocyte transformation test and by determination of blast indices (p.52 and p.48).

In Vitro Response by SMC from Infected rats to Intact *D.congolensis* Cocci

As an alternative to looking at the *in vitro* response of SMC from infected rats to *D.congolensis* antigen, the response to intact, but inactivated, *D.congolensis* cocci was examined.

Inactivation of *D.congolensis*

Dermatophilus congolensis was rapidly inactivated by the concentrations of penicillin and streptomycin which were routinely included in the medium for SMC cultures (see p.67).

D.congolensis cocci were harvested in PBS from 48 hour B/A plate cultures. Aliquots were taken for viable counts (Appendix 1), for Gram stains and for streaking out on B/A plates, the latter being purity checks. Ten-fold dilutions of the remaining suspension were made in complete medium and these were incubated for one hour at 37°C prior to their addition to the cultures, such that only inactivated cocci were incubated with SMC.

As a further check that the *D.congolensis* cocci did not play an active role in the SMC cultures, wells containing cocci and medium but lacking in SMC were included in the experiments. At the end of the culture period, it could be demonstrated that no ^{3}H-thymidine uptake occurred in the absence of SMC (p.67).

Blast Indices and Lymphocyte Transformation Test

SMC were isolated on the tenth day after the final *D.congolensis* infection and on the tenth day after the final PPD injection or scarification for controls (p.52). The cells were cultured as previously described for five days, with half the medium replenished with fresh on day three (p.52). Seven different treatments were tested; these were

addition of *D.congolensis* cocci at five different dilutions, addition of PPD at 20 μg ml^{-1}, or no additions to the complete medium. For each treatment, eight replicates were set up for the lymphocyte transformation test and three additional replicates for cytospins; these were carried out on day five by the methods described previously (p.52 and p.48).

RESULTS

MONONUCLEAR CELL ISOLATION FROM BLOOD

Usually, 10-12 ml blood could be collected when an adult rat was exsanguinated. The mean leucocyte count (five Wistar males) was 10.70×10^6 cells ml^{-1}. After separation of 10 ml of blood on a Ficoll gradient, a total of approximately 2.5×10^7 MC were harvested. This suggests an apparent yield of 24 per cent. However, the true yield of MC was 31 per cent, since MC represent only about 75 per cent of the leucocyte count of whole blood for rats of the same age and sex as used here (Schalm, Jain and Carroll 1975). The yield was not great enough for the envisaged experiments, therefore, an alternative source of cells was investigated.

MONONUCLEAR CELL ISOLATION FROM SPLEEN

Depending on the size of the rat, an average of 5.7×10^8 leucocytes were harvested per spleen (mean of 87 spleens). Differential counts of Giemsa-stained cytospin preparations showed that erythrocytes were the most numerous cell type, followed by lymphocytes, monocytes and then PMN (Figure 2.1, table 2.1). Eosinophils were observed at a frequency of less than one per cent. Haemopoeitic stem cells were not differentiated from the other splenocytes. The distribution of the cell types was essentially similar for splenocytes obtained from normal rats, from rats infected with *D.congolensis* and from rats sensitised to the antigen PPD (Table 2.1).

The optimum centrifugal force for separation of rat SMC on a Ficoll gradient was found to be 480g at the interface, giving a yield of 30 per cent of the MC originally layered on the gradient (Appendix 1).

Table 2.1: Percentage cell types in normal, infected and antigen-sensitised rat spleen suspensions

Cell type	Non-sensitised	*D. congolensis*-infected	PPD-sensitised
Erythrocyte	63 (56-84)	70 (48-87)	65 (62-79)
Small lymphocyte	34 (13-40)	25 (12-43)	25 (17-32)
Blast lymphocyte	1 (0-1)	1 (0-2)	1 (0-3)
Monocytes / macrophage	1 (1-4)	2 (1-5)	3 (2-7)
Polymorphonuclear	1 (0-1)	1 (0-2)	2 (1-3)
	n=6	n=14	n=5

Values are medians with the range given in brackets, n represents the number of times carried out.†

Table 2.2: Percentage cell types in normal, infected and antigen-sensitised rat splenocytes separated on a Ficoll gradient.

Cell type	Non-sensitised	*D. congolensis*-infected	PPD-sensitised
Small lymphocyte	92 (89-95)	94 (88-98)	88 (87-93)
Blast lymphocyte	2 (0-4)	1 (0-4)	2 (1-2)
Monocytes / macrophage	4 (3-4)	3 (0-9)	6 (3-9)
Erythrocyte	2 (1-5)	2 (1-4)	2 (1-5)
Polymorphonuclear	0 (0-1)	0 (0-1)	0 (0)
	n=6	n=14	n=5

Values are medians with the range given in brackets, n represents the number of times carried out.†

† Cells were isolated ten days after initiation of the third infection or sensitisation with antigen.

The cells harvested from the gradients were heavily contaminated with platelets and several different washing times and speeds were tested to remove them. Two five minute washes at 300g removed the majority of platelets and was routinely used, even though this resulted in a substantial loss of cells.

The mean number of MC finally obtained per spleen was 9.1×10^7 cells, with an overall yield of 18 per cent (n=80). Giemsa-stained cytospins revealed that most erythrocytes, PMN and platelets had been removed, leaving lymphocytes and monocytes (Figure 2.2). The distribution of cell types is given in table 2.2, which also shows the distribution for cells from *D.congolensis*-infected and PPD-sensitised rats. Again, the three different treatments made no difference to the differential counts.

The viability of the separated mononuclear cells ranged from 96 to 99 per cent, with a median value of 98 per cent (n=9).

RESPONSE OF SPLEEN MONONUCLEAR CELLS TO THE ANTIGEN PPD

Skin Testing

Negative skin reactions to PPD were obtained for rats previously injected with PPD by the subcutaneous route with an increase in skin thickness over 48 hours which was no greater than that of controls. In contrast, positive skin reactions occurred with the rats previously injected with PPD intra-dermally, with noticeable erythema visible in the test rats after 24 hours, whereas, only one of the control rats showed erythema, which was very mild, also at 24 hours. The skin thickness of both test and control rats increased after injection of PPD, but the increase in the test rats was twice that of the controls (Table 2.3). Although a further control of intradermal injection of the vehicle alone (PBS) was not included in these tests, such controls have been included

in numerous skin tests carried out in this laboratory. In no case was there an increase in skin thickness greater than 0.05 mm.

SMC Response to PPD *In Vitro*

Regardless of whether the rat had been sensitised to PPD by the intra-dermal or the subcutaneous route, isolated SMC responded well to PPD *in vitro*, with a high proportion of lymphoblasts often becoming noticeable on day three (Figure 2.3). In contrast, fewer lymphoblasts were observed when the SMC derived from a non-sensitised rat (Figure 2.4) or when SMC from either source were cultured in the absence of PPD. The percentage lymphoblasts in cultures containing PPD was significantly greater when the SMC derived from PPD-sensitised rats than from non-sensitised rats (Table 2.4, Mann-Whitney $U_4^4=0$, $P < 0.05$).

Since a certain amount of background stimulation is always present and varies with the batch of FCS and culture conditions, this factor was removed from the results by subtracting the corresponding blast index of cells cultured in medium alone from that of cells cultured with antigen (Figure 2.5).

The cell concentrations in the cultures derived from PPD-sensitised rats increased by a factor of 1.8 in the presence of PPD; whereas the concentration in cultures derived from non-sensitised rats reached a maximum of 1.3 times the initial value (Table 2.5).

Table 2.3: Skin thickness of test sites after intradermal injection of PPD

Animal	Thickness (mm)			
Time (h)	0	24	48	Max.
PPD-sensitised rat 1	0.58	0.79	0.78	0.21
PPD-sensitised rat 2	0.61	0.85	0.84	0.24
Non-sensitised rat 1	0.71	0.80	0.76	0.09
Non-sensitised rat 2	0.62	0.73	0.66	0.11

Max. represents the maximum increase in mm from time 0.

Table 2.4: Median blast indices of SMC cultured with various concentrations of PPD

PPD	Blast index (%)					
($\mu g\ ml^{-1}$)	Non-sensitised rat			PPD-sensitised rat		
Time (d)	3	5	7	3	5	7
0	28	34	43	22	39	41
5	26	41	45	33	72	70
10	19	44	50	30	80	79
20	29	44	48	34	87	85
Con A	98	98	96	97	98	98

The values are medians of 4 replicate cultures. At least 200 cells were counted each time. The indices for the PPD-sensitised rat are significantly greater than those of the control rat for 5, 10 and 20 $\mu g\ ml^{-1}$ PPD on days 5 and 7 (Mann-Whitney $U_4^4=0$, $P < 0.05$).

Table 2.5: Cell counts of SMC cultured with various concentrations of PPD

PPD ($\mu g\ ml^{-1}$)	Cells x $10^6\ ml^{-1}$					
	Non-sensitised rat			PPD-sensitised rat		
Time (d)	3	5	7	3	5	7
0	1.1	1.2	0.9	1.1	1.0	0.7
5	1.3	1.2	1.2	1.0	1.3	1.2
10	1.0	1.1	0.9	1.0	1.5	1.2
20	1.0	1.0	1.2	1.1	1.8	1.6
Con A	2.7	3.0	3.9	2.0	4.0	3.4

Values given are means of duplicate samples. The initial cell concentration was $1x10^6\ ml^{-1}$

THE RESPONSE OF SPLEEN MONONUCLEAR CELLS TO *DERMATOPHILUS CONGOLENSIS*

RESPONSE TO *D.CONGOLENSIS* ANTIGEN BY SMC FROM ANTIGEN-SENSITISED RATS

Skin Testing

Rats in the experimental groups which had been administered either filamentous or coccoid-form antigen intra-dermally, were skin tested with the antigen on the same day that SMC were isolated from the remaining rats in the groups.

Following intra-dermal administration of 50 μg *D.congolensis* coccoid antigen, ten days after initial exposure to the antigen, an increase in skin thickness occurred at the site of injection which was 1.9 times greater in the rats sensitised to *D.congolensis* antigen than in the

controls (Table 2.6); the difference however was not significant at the five per cent level (t-test, $0.10 > P > 0.05$).

Table 2.6: Skin thickness of test sites after intradermal injection of *D. congolensis* coccoid antigen

Animal	Thickness (mm)				
Time (h)	0	24	48	Max.	
Ag-sensitised rat 1	0.81	0.91	0.84	0.10	
Ag-sensitised rat 2	0.48	0.65	0.64	0.17	(0.15 +/- 0.05)
Ag-sensitised rat 3	0.56	0.75	0.70	0.19	
Non-sensitised rat 1	0.69	0.76	0.72	0.07	
Non-sensitised rat 2	0.56	0.65	0.65	0.09	(0.08 +/- 0.01)
Non-sensitised rat 3	0.61	0.70	0.65	0.09	

Ag represents antigen, Max. represents the maximum increase from day 0. Values in brackets are the mean and standard deviation.

The filamentous antigen gave inconclusive skin test results when tested on rats ten days after the final infection. Three out of four sensitised rats showed thickness increases of 0.07 to 0.08 mm, whilst the fourth animal did not react at all. Three out of four non-sensitised rats showed thickness increases of 0 to 0.02 mm, but the fourth showed an increase of 0.10 mm.

In Vitro Response to *D. congolensis* Antigen by SMC from Antigen-Sensitised Rats

SMC isolated from rats which had been given one 150 µg dose of coccoid form antigen, showed greater blast indices than did SMC from naive rats,

~~from naive rats~~, when exposed to the antigen *in vitro* (Mann-Whitney $U_4^4=0$, $P < 0.05$) except at the lowest dose of antigen. However, the response was comparatively weak, with blast indices which were only slightly higher than those for the same cells cultured with PPD (Table 2.7).

Table 2.7: Median blast indices of *D.congolensis* coccoid antigen-stimulated SMC with background stimulation subtracted

Concentration		Blast index (%)					
antigen		Non-sensitised rat			Ag-sensitised rat		
($\mu g\ ml^{-1}$)	Time (d)	3	5	7	3	5	7
2		8	0	6	5	7	8
20		11	4	6	21	9	16
200		14	9	10	20	19	22
PPD		-	-	-	13	14	22

Ag represents *D.congolensis* coccoid antigen. PPD was included at 20 $\mu g\ ml^{-1}$. The control rat sensitised to PPD gave corresponding blast indices of 26, 47 and 50 on days 3, 5 and 7 respectively when cultured with PPD. Values are medians of 4 replicate cultures.

SMC isolated from rats which had been given three doses of 150 μg filamentous antigen, responded even more weakly, despite being incubated with higher doses of antigen, ie. 30, 300 and 3,000 $\mu g\ ml^{-1}$, than in the previous experiment. The *D.congolensis* antigen was tested at dry weight concentrations, which included one of the same order of magnitude as that of PPD. Although *D.congolensis* antigen contained far less protein than PPD, it was tested at a protein concentration which was just half that of PPD (Table 2.8).

Table 2.8: Dry weight and protein concentrations of the antigens used in SMC cultures

Antigen	Dry weight concentration ($\mu g\ ml^{-1}$)	Protein concentration ($\mu g\ ml^{-1}$)
DC	30	0.07[1]
DC	300	0.70[1]
DC	3,000	7.00
PPD	20	15.00

DC represents D.congolensis filamentous-form antigen.

[1]Extrapolated value.

RESPONSE TO D.CONGOLENSIS ANTIGEN AND COCCI BY SMC FROM INFECTED RATS

Determination of Labelling Conditions for Lymphocyte Transformation Test

The amount of ^{3}H-thymidine uptake by SMC was proportional to the amount of added label and the incubation period, for cells cultured in medium alone (Figure 2.6) or with *D.congolensis* (Figure 2.7).

Saturation conditions were not obtained under the conditions tested (Figures 2.6 and 2.7). As the amount of label is increased and the pulse time is decreased, a point should be reached where the system is saturated. Here the uptake increased with increasing amounts of label but this did not reach a plateau. A decrease in uptake was seen with the higher amounts of ^{3}H-thymidine when incubated for 16 hours which could indicate radiation damage of the SMC.

Stimulation indices (SI) were derived from the ^{3}H-thymidine values:

$$SI = Md_{DC} / Md_{M} \quad (1)$$

Where Md_{DC} represents the median CPM of SMC cultured with *D.congolensis* cocci and Md_{M} represents the median CPM of SMC cultured in medium alone.

Despite the wide range of CPM values under different labelling conditions, the stimulation index remained relatively constant (Table 2.9).

Table 2.9: Stimulation indices of SMC under various labelling conditions

^{3}H-thymidine	Pulse time (h)			
(µCi / well)	2	4	8	16
0.125	6.2	6.0	4.1	3.9
0.25	5.7	6.4	4.3	5.1
0.50	7.4	6.5	6.8	4.7
1.00	6.3	6.0	7.5	5.9
2.00	7.0	9.1	5.7	4.5

Values are derived from medians of 8 replicates.

In Vitro Response by SMC from Infected Rats to *D.congolensis* Antigen

SMC isolated ten days after the final infection was induced, failed to respond to *D.congolensis* filamentous-form antigen *in vitro* over the range of 0.1 µg ml^{-1} to 10 mg ml^{-1}, as assessed by the lymphocyte transformation test and blast index counts. A surprising exception to

this was a significant response to the antigen at 1 mg ml^{-1}, with a stimulation index of four (median CPM=18,000 compared with 4,000 CPM for the control, Mann-Whitney $U_4^4=0$, $P < 0.05$) and a blast index of 50 per cent (12 per cent once background subtracted). However, this result was only obtained in two out of three experiments.

In Vitro Response by SMC from Infected Rats to Intact *D.congolensis* Cocci

Inactivation of *D.congolensis*

The concentrations of antibiotics routinely used in the medium for SMC culture were sufficient to rapidly inactivate *D.congolensis*. Growth, assessed by incubation on B/A plates, was completely inhibited following one hour of incubation at 37°C with complete RPMI medium containing 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Pencillin and streptomycin at half the concentration used ie. 50 units/µg ml^{-1} were equally as effective. In contrast, *D.congolensis* grew well when incubated with the complete medium in the absence of antibiotics. That the penicillin and streptomycin were bacteriocidal to *D.congolensis*, rather than bacteriostatic, was demonstrated by the absence of growth even when the cocci were thoroughly washed following the incubation with antibiotic-containing medium.

An essential control in experiments involving intact *D.congolensis* cocci was to include some wells in the lymphocyte transformation test which contained all the constituents bar SMC. Thus, any uptake by *D.congolensis* itself would be detected. In no cases, was uptake of ^3H-thymidine by *D.congolensis* demonstrated. The CPM of these wells lay within the normal range for background radiation, determined in each experiment. A typical median value of CPM with *D.congolensis* cocci at $2x10^6$ ml^{-1} was 36 (range 28-54), whilst the corresponding median value of

CPM for SMC with no label added (background) was 30 (range 15-100); eight replicates of each were set up.

Blast Indices

A high proportion of lymphoblasts were observed in day five Giemsa-stained cytospins of SMC derived from *D.congolensis*-infected rats and cultured with $8x10^6$ cocci ml^{-1} (Figure 2.8). In contrast, SMC from the same rat, cultured in medium alone were mostly small lymphocytes (Figure 2.9). Likewise, SMC derived from non-sensitised rats and cultured with $8x10^6$ cocci ml^{-1} for five days were mostly small lymphocytes (Figure 2.10). Thus, culture with *D.congolensis* caused a high degree of blast transformation of SMC from infected rats only.

Table 2.10 shows the blast indices of the SMC cultures from test and control rats, incubated with a range of *D.congolensis* cocci concentrations. The extremely high blast indices of SMC from infected rats, when stimulated with *D.congolensis in vitro*, is partially explained by the high background blast response. Table 2.11 gives the blast indices once the background has been subtracted. The cells from the infected rat showed a dose-dependent response to *D.congolensis* cocci, up to a maximum value with $8.2x10^6$ ml^{-1} ($\log_{10}$ 6.9). Non-specific stimulation by *D.congolensis* also occured with high blast indices of SMC from naive and PPD-sensitised rats, when cultured with the cocci but the blast indices of these cultures were always much lower than those of SMC from infected rats and the ranges did not overlap.

Table 2.10: Day five blast indices of SMC cultured with various concentrations of *D.congolensis* cocci

D.congolensis	Percentage blast cells		
$\log_{10}$ ml^{-1}	Non-sensitised rat	*D.congolensis*-infected rat	PPD-sensitised rat
0	10 (8-20)	32 (28-42)	14 (14-18)
3.9	18 (14-19)	54 (38-54)	15 (15-16)
4.9	22 (18-22)	81 (78-83)	24 (19-36)
5.9	48 (43-54)	90 (87-97)	36 (30-47)
6.9	45 (42-52)	92 (87-93)	54 (48-56)
7.9	28 (22-32)	82 (82-84)	34 (34-36)
PPD	25 (18-32)	60 (58-60)	48 (48-56)

Values are medians of three replicates. The range is given in brackets. PPD was at 20 µg ml^{-1}.

Table 2.11: Day five blast indices of SMC cultured with various concentrations of *D.congolensis* cocci with background subtracted

D.congolensis	Percentage blast cells		
$\log_{10}$ ml^{-1}	Non-sensitised rat	*D.congolensis*-infected rat	PPD-sensitised rat
3.9	8	22	1
4.9	12	49	10
5.9	38	58	22
6.9	35	60	40
7.9	18	50	20
PPD	15	28	34

Values are medians of three replicates. PPD was at 20 µg ml^{-1}.

Lymphocyte Transformation Test

Table 2.12 shows the results from the lymphocyte transformation test, with CPM of SMC from test and control rats, cultured with a range of concentrations of *D.congolensis* cocci. The stimulation indices for this experiment are illustrated in figure 2.11.

When SMC were cultured *in vitro* with *D.congolensis* cocci, a degree of stimulation occurred regardless of the origin of the cells. However, the ^{3}H-thymidine uptake by the cells from the *D.congolensis*-infected rat was significantly greater than that by the cells from the naive control, for all concentrations of *D.congolensis* cocci (Mann-Whitney U_{8}^{8}=7, $P < 0.01$ for $\log_{10}$ 7.9, 6.9, 5.9, 4.9 and $P < 0.05$ for $\log_{10}$ 3.9). The CPM of SMC from the infected rat were also significantly greater than those from the PPD-sensitised rat over most of the concentrations of cocci (Mann-Whitney U_{8}^{8}=7, $P < 0.01$ for $\log_{10}$ 6.9, 5.9, 4.9).

There was some cross reaction between the PPD antigen and the *D.congolensis* cocci. PPD caused significant stimulation of SMC from infected rats, which responded far more strongly to it than did SMC from naive rats (Mann-Whitney U_{8}^{8}=7, $P < 0.01$). However, this cross-reaction was uni-directional, since SMC from a PPD-sensitised rat did not respond more stongly to the cocci than did cells from a naive rat, except for at the two extreme concentrations of $\log_{10}$ 3.9 and 7.9 (Mann-Whitney U_{8}^{8}= 13, $P < 0.05$).

In two repeat experiments, SMC from *D.congolensis*-infected rats always gave stimulation indices which were significantly greater than those of controls, when cultured with *D.congolensis* cocci (Mann-Whitney U_{8}^{8}=7, $P < 0.01$). The actual values of the stimulation indices varied from one experiment to the next. The highest SI occurred with *D.congolensis* cocci at concentrations of 6-27x10^{6} ml^{-1}; these values for the three

experiments were 185, 172 and 10. The wide range of SI was largely due to the 100-fold variation, between different experiments, in the ^{3}H-thymidine uptake by the non-stimulated cells cultured in medium alone.

Table 2.12: ^{3}H-thymidine uptake by SMC on day five of culture with various concentrations of *D.congolensis* cocci

D.congolensis	Median CPM x 10^{3}					
$\log_{10}$ ml^{-1}	Non-sensitised rat		*D.congolensis*-infected rat		PPD-sensitised rat	
0	6	(4-10)	8	(6-12)	3	(2-4)
3.9	6	(3-9)	19	(14-40)	6	(5-10)
4.9	14	(10-23)	66	(59-75)	9	(8-16)
5.9	14	(11-22)	68	(58-77)	10	(8-13)
6.9	22	(13-34)	87	(66-99)	12	(8-18)
7.9	1	(0)	9	(5-14)	4	(1-8)
PPD	8	(4-9)	47	(38-53)	31	(21-37)

Values are medians of eight replicates rounded up to the nearest thousand. The range is given in brackets. PPD was at 20 μg ml^{-1}.

DISCUSSION

A convenient and plentiful source of rat mononuclear cells (MC) was essential for an *in vitro* study of the response of these cells to *D.congolensis*. The first source investigated was blood withdrawn by exsanguination. About $2x10^7$ MC were obtained from one rat following separation of blood on a Ficoll gradient. Although the yield of MC could probably be improved by refinement of the separation procedure, it was unlikely to reach more than 30-50 per cent (Brown 1987). It became apparent that insufficient quantities of cells would be available for the experiments envisaged.

The next source of MC investigated was the spleen. The method of density gradient centrifugation employed, yielded a MC population with high viability, which was virtually free of polymorphonuclear cells (PMN) and eosinophils and contained only a few erythrocytes. Almost five times the number of MC could be obtained from the spleen as from the blood from one rat. A greater yield could have been obtained with higher centrifugation forces when washing the cells. However, even though 12 per cent of cells were lost during the washes, the end product was essentially free of platelets. This compromise between yield and purity was worthwile, since platelets, carried over into a MC culture, soon die off, releasing toxic substances which could adversely affect the mononuclear cells. The same is true for PMN which would degenerate within a few days (Ling and Kay 1975).

Mature MC recirculate continuously between the blood and the lymphoid organs. The spleen is the predominant organ in MC recirculation with about 8×10^7 lymphocytes exchanged between it and the blood each hour in the rat (Ford 1969) and with a greater circulation rate than that of all

the lymph nodes put together (Pabst, cited by Pabst 1988). The spleen was therefore chosen as a rich and convenient source of MC for this study.

A well-documented phenomenon in the field of lymphocyte biology is lymphocyte stimulation, also known as lymphocyte activation or blast transformation; these refer to the morphological (see p.48) and metabolic changes which occur when lymphocytes are exposed to certain lectins (mitogens), to allogeneic lymphocytes, or to antigens to which previous exposure has occurred. One of the most obvious changes is that a high proportion of cells are stimulated to enter mitosis and one of the most frequently assessed metabolic changes is that of the rate of thymidine uptake (Ling and Kay 1975).

Unlike mitogens, antigens only induce significant lymphocyte stimulation when the animal, from which the cells originated, has already been exposed to that antigen (Ling and Kay 1975). As a preliminary to investigating the response of rat SMC to *D.congolensis* antigen after *in vivo* exposure, the response to a known antigen, PPD, was studied. Flax and Waksman (1962) used skin testing with PPD as a measure of the state of sensitisation of Wistar rats. They claimed that the intra-dermal route was the most effective for sensitisation to inactivated *Mycobacterium tuberculosis*, whereas subcutaneous inoculation was relatively ineffectual. In this study, Wistar rats injected intra-dermally with PPD gave positive skin tests, while those injected subcutaneously gave negative skin tests. However, SMC from both intradermally and subcutaneously sensitised rats underwent blast transformation *in vitro* in response to 20 $\mu g\ ml^{-1}$ PPD. The response to PPD was specific, requiring prior sensitisation, since the blast indices of SMC from naive rats, in the presence of PPD, were significantly lower than those of SMC from sensitised rats ($P < 0.05$).

An effective method of sensitisation having been found for the antigen PPD, the same protocol was then used for sensitisation of rats to *D.congolensis* antigen. Senstisation of rats to *D.congolensis* coccoid antigen preparation induced positive skin tests and a blast response to the antigen *in vitro* (Tables 2.6 and 2.7).The blast response, although greater than that of naive SMC, was not as strong as might have been expected from the response of PPD-sensitised SMC to PPD. Also, some cross-reaction with PPD occurred with the rats sensitised to *D.congolensis*, giving blast responses to PPD which were comparable with those to *D.congolensis* coccoid antigen.

The response to a different antigen preparation was next examined. The method of antigen preparation was the same, but the growth stage of *D.congolensis* used was the filamentous stage. Also, the rats were given three doses of the antigen over a period of thirty days, since repeated doses may be necessary to induce sensitisation. The blast response was, however, weaker than that obtained with coccoid antigen.

There are several possible reasons why such a poor response was obtained to both *D.congolensis* antigens. The antigens may have failed to elicit a response because the preparation was too crude, containing many impurities which might swamp the stimulatory properties of fractions which were perhaps present at very low levels. Wikel, Graham and Allen (1978) found that only low doses of a crude antigen preparation of tick salivary gland antigen were stimulatory to sensitised lymphocytes *in vitro*. Purification of the *D.congolensis* antigen was not undertaken, but a large range of concentrations were tested in the SMC cultures to allow impurities to be diluted out.

Insufficient quantities of potentially immunogenic components in the preparation was possibly not the cause of the lack of reaction. It is the

protein fraction, rather than the carbohydrate component, of *Mycobacterium tuberculosis* which induces lymphocyte transformation (Janicki, Aron, Schechter and Mc Farland 1972). Although the same is not necessarily true for *D.congolensis*, the range of antigen concentrations tested did include one with a comparable protein content to PPD (Table 2.8).

One possibility was that the route of administration may have been an ineffective one for *D.congolensis* antigen. However, when rats were subjected to experimentally-induced *D.congolensis* infection, rather than to injection with antigen, no *in vitro* response to the antigen occurred, except for one concentration of antigen. The blast and thymidine uptake response to this one concentration has to be dismissed because it only occurred on two of three occassions and because there was no evidence of the expected dose response to a range of concentrations.

The most likely explanation for the poor response is that the method of antigen preparation disrupted any potentially immunogenic determinants. In general, antigens which induce a delayed hypersensitivity as opposed to a humoral response are usually whole bacteria or large protein complexes (Bullock 1978). Although *D.congolensis* antigen has been used successfully, in our laboratory, in the detection of circulating antibodies to *D.congolensis* in infected animals this does not necessarily predict that the same antigen preparation will be capable of activating lymphocytes. In support of this view, borderline leprosy patients with predominantly cutaneous lesions have been observed to respond well to whole *Mycobacterium leprae* in the lymphocyte transformation test but only weakly to the sonicated bacilli (Barnetson, Bjune, Pearson and Kronvall 1976).

Only one published report has investigated the *in vitro* lymphocyte

response to *D.congolensis* antigen by cells from infected animals. Makinde and Wilkie (1979) studied the response of leucocytes from experimentally-infected rabbits, at five-day intervals, to crude *D.congolensis* antigen in a lymphocyte transformation test. The results were rather surprising; although a response to the antigen did occur it was only observed on days ten and twenty, with a complete failure to respond on day fifteen, or on the other days tested. Only derived data was presented so it is impossible to assess important factors which control the reliability of the test, such as the state of the cells. Indeed, the cell viability may have been poor since the test was carried out six days after initiation of culture, with no replenishment of fresh medium during this time. Furthermore, with the method used for isolation of lymphocytes from blood, from six to thirty per cent of PMN were likely to be present in the cultures (Thomson, Bull and Robinson 1966); PMN die off quickly in culture with the release of toxic products (Ling and Kay 1975). In support of this criticism, very poor responses to mitogens were obtained with leucocytes from non-infected rabbits, with stimulation indices of three for PHA and just over one for lipopolysaccharide, whereas high stimulation indices would normally be expected.

Since the response of SMC to *D.congolensis* antigen preparations was weak, the response to intact *D.congolensis* cells was investigated. The study was restricted to the coccoid stage of growth since the cocci are readily quantifiable, unlike the filamentous stage of the life cycle. In either an experimental or a natural infection, the host would be exposed to both the coccoid and the filamentous stages, so it is reasonable to use either one as the stimulant in the SMC cultures. It was essential to first ensure that the *D.congolensis* cocci were not viable and could not play an active role in the SMC cultures but were merely inert antigenic

structures. The cocci were inactivated prior to addition to the cultures, by incubation in medium containing the antibiotics routinely used in SMC cultures. A further check was that *D.congolensis* incubated under the same culture conditions as SMC, did not incorporate ^{3}H-thymidine.

The response of SMC from infected rats to intact cocci of *D.congolensis* was assessed by means of the lymphocyte transformation test and by determination of blast indices. Before the ^{3}H-thymidine incorporation, lymphocyte transformation test could be employed, the appropriate labelling conditions had to be determined. Sample and Chretien (1971) pointed out that a number of factors affect both the rate of thymidine incorporation and how accurately this rate reflects the rate of deoxyribonucleic acid (DNA) synthesis. For example, the size of the endogenous thymidine pool will affect both the rate of uptake and the rate of DNA synthesis. The effect of these factors is minimised when large enough amounts of exogenous, labelled thymidine are added to saturate the system ie. such that the exogenous is far greater than the endogenous pool. Under saturation conditions, addition of further amounts of ^{3}H-thymidine will not cause increased uptake.

Despite the wide range of ^{3}H-thymidine concentrations and incubation times tested, saturation conditions were not obtained in this study (Figures 2.6, 2.7). Nevertheless, the amount of label and incubation time had little effect on the stimulation index, which is the parameter of interest (Table 2.9). Furthermore, for reasons of cost alone, it would be impractical to increase the ^{3}H-thymidine concentration above those tested. At the higher concentrations, the amount of uptake actually began to decrease with the longest incubation time of sixteen hours (Figures 2.6, 2.7). This suggests either radiation damage or degradation of some of the incorporated label. The concentration of ^{3}H-thymidine used, which

was that giving 0.5 µCi/well, was chosen to ensure reasonable counts, since in some studies the rate of uptake of control cultures was expected to be low. The incubation period used was chosen because longer periods increased the risk of radiation damage and with shorter periods, fewer cells would be in the DNA-synthesis phase (S-phase) when exogenous thymidine is incorporated (Sample and Chretien 1971).

Spleen mononuclear cells derived from infected rats gave a strong and specific response to *in vitro* stimulation with *D.congolensis*, as shown by blast indices (Table 2.11) and the lymphocyte transformation test (Figure 2.11). The experiment was repeated twice more with the same result. There was, however, a large amount of variability in the stimulation indices obtained in the three experiments although this did not affect the significance (p.70). The variation was largely a reflection of the differences in background uptake between experiments.

The main cause of background stimulation is usually FCS proteins in the culture medium (Ling and Kay 1975). Although the same batch of FCS was always used within an experiment, different batches were sometimes used for other experiments; this may explain some of the observed variation in background uptake. In addition, the spleen is known for its high background incorporation of thymidine compared with lymphocytes from the blood (Hudson and Hay 1976); it would appear that the uptake rate varies markedly from one rat to the next. One consequence of the high background stimulation was that SMC from infected rats gave much higher blast indices, when cultured with the cocci, than might be expected for an antigen as opposed to a mitogen induced response (Table 2.10). It was only when the background stimulation was subtracted (Table 2.11) that indices which lie within the expected range were produced.

An interesting result was the significant stimulation ($P < 0.01$) of SMC

from *D.congolensis*-infected rats by the antigen PPD (Figure 2.11). One can speculate that some common antigens may be shared by *D.congolensis* and *Mycobacterium tuberculosis*, to bring about this cross-reaction. Indeed, the two organisms are distantly related, both are actinomycetes belonging to different families within the same group (Brock 1979). If so, it is perhaps, surprising that *D.congolensis* cocci did not cause significant stimulation of SMC from PPD-sensitised rats compared with naive rats.

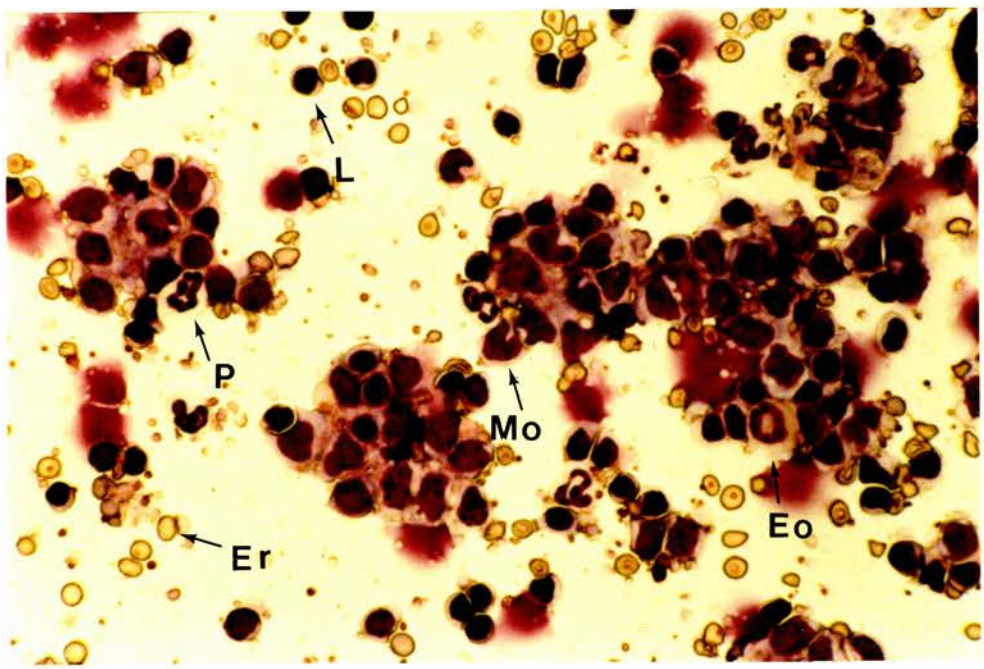
The results indicate that experimentally-induced infection of rats with *Dermatophilus congolensis* primes a population of mononuclear cells *in vivo*, which will subsequently respond to *D.congolensis* cocci *in vitro*. The immuno-phenotype of the responding *in vitro* population was then determined by the method described in the next chapter.

FIGURE 2.1 Unpurified rat spleen mononuclear cells

Giemsa-stained cytospin showing:
Er erythrocyte
L lymphocyte
Mo monocyte
P polymorphonuclear leucocyte
Eo eosinophil
(x 425 magnification)

FIGURE 2.2 Rat spleen mononuclear cells harvested from a Ficoll density gradient. The majority of cells are small lymphocytes together with some monocytes. Erythrocytes, polymorphonuclear leucocytes and eosinophils are virtually absent. Giemsa-stained cytospin.

(x 425 magnification)


L
P
Mo
Eo
Er

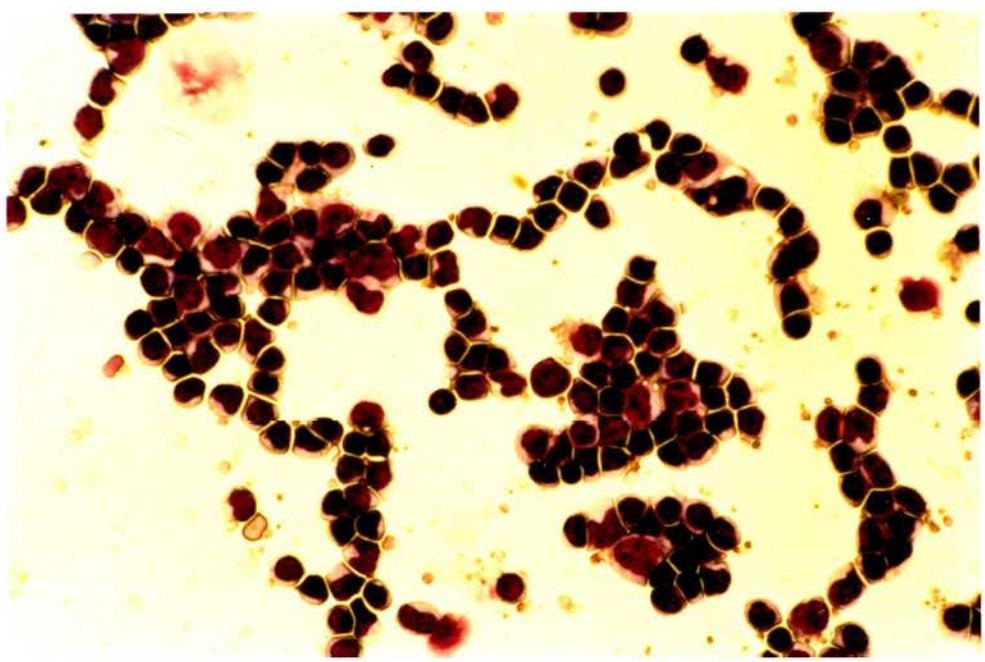
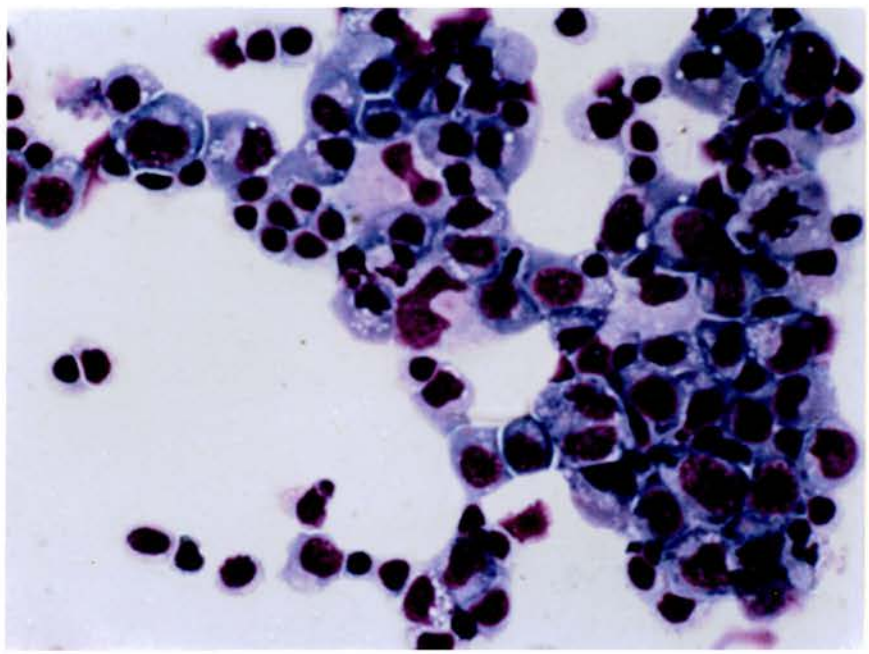


FIGURE 2.3 Spleen mononuclear cells derived from a PPD-sensitised rat and cultured for three days in the presence of 20 $\mu g\ ml^{-1}$ PPD. Many of the lymphocytes are blastoid. Giemsa-stained cytospin.

(x 425 magnification)

FIGURE 2.4 Spleen mononuclear cells derived from a non-sensitised rat and cultured for three days in the presence of 20 $\mu g\ ml^{-1}$ PPD. Few blastoid cells are present. Giemsa-stained cytospin.

(x 425 magnification)



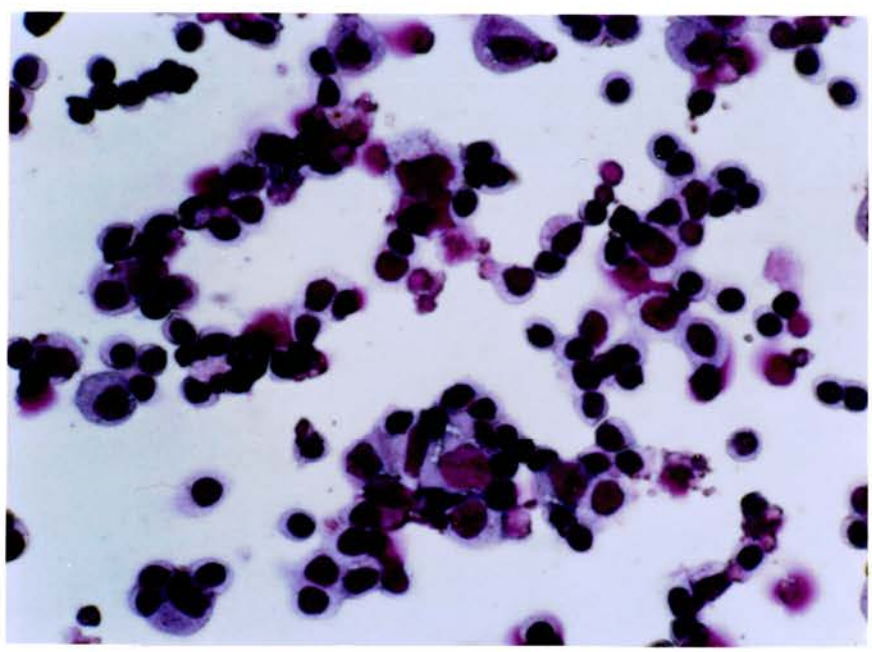


FIGURE 2.5 Median blast indices of spleen mononuclear cells cultured for five days with various concentrations of PPD or with 2 $\mu g\ ml^{-1}$ concanavlin A. Background stimulation, assessed by the blast index of cells cultured in medium alone, has been subtracted. The blast indices of SMC from the sensitised rat are significantly greater than those of SMC from the non-sensitised rat at all concentrations of PPD (Mann-Whitney $U_4^4=0$, $P < 0.05$)

SMC from a non-sensitised rat

SMC from a PPD-sensitised rat

BLAST INDEX %
0
20
40
60
80
5
10
20
ConA
PPD CONCENTRATION (μg/ml)

FIGURES 2.6 and 2.7 Determination of labelling conditions for ^{3}H-thymidine incorporation lymphocyte transformation test.

Counts per minute of SMC derived from infected rats pulsed with various concentrations of, and for different times with, ^{3}H-thymidine.

FIGURE 2.6 SMC cultured in medium alone

FIGURE 2.7 SMC cultured with an optimal concentration of *D.congolensis* inactivated cocci.

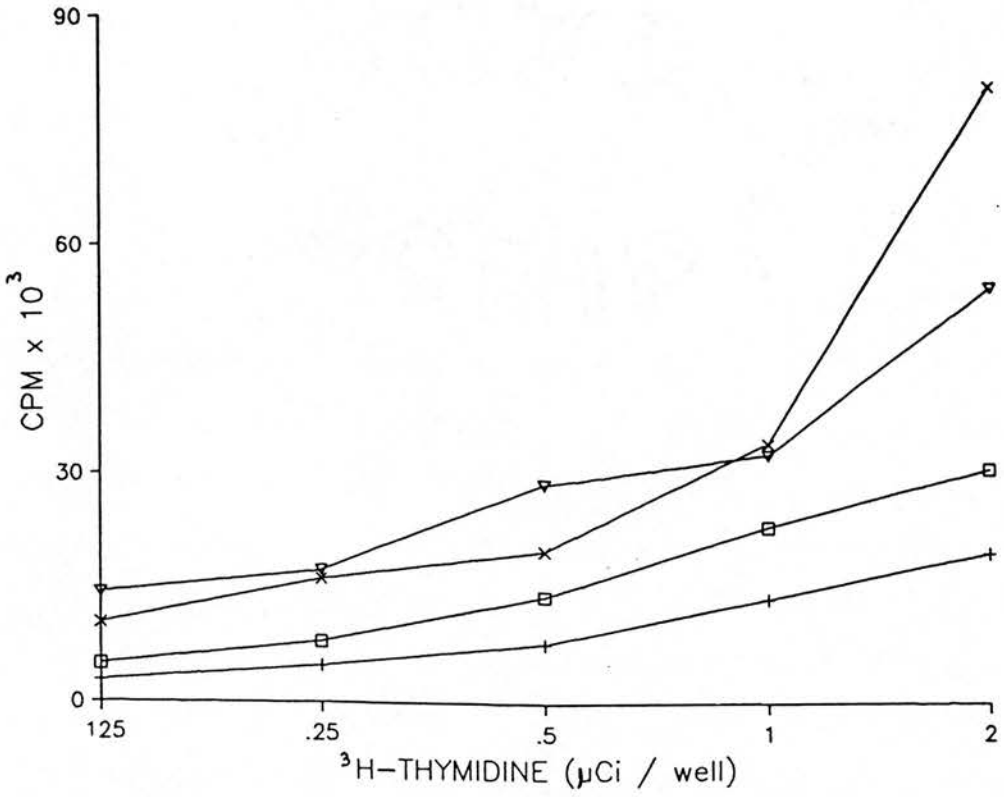
+ 2 hour pulse

□ 4 hour pulse

× 8 hour pulse

▽ 16 hour pulse

Saturation conditions were not attained under any of the conditions tested.


90
60
30
0
CPM x 10^3
125
.25
.5
1
2
^{3}H–THYMIDINE (µCi / well)

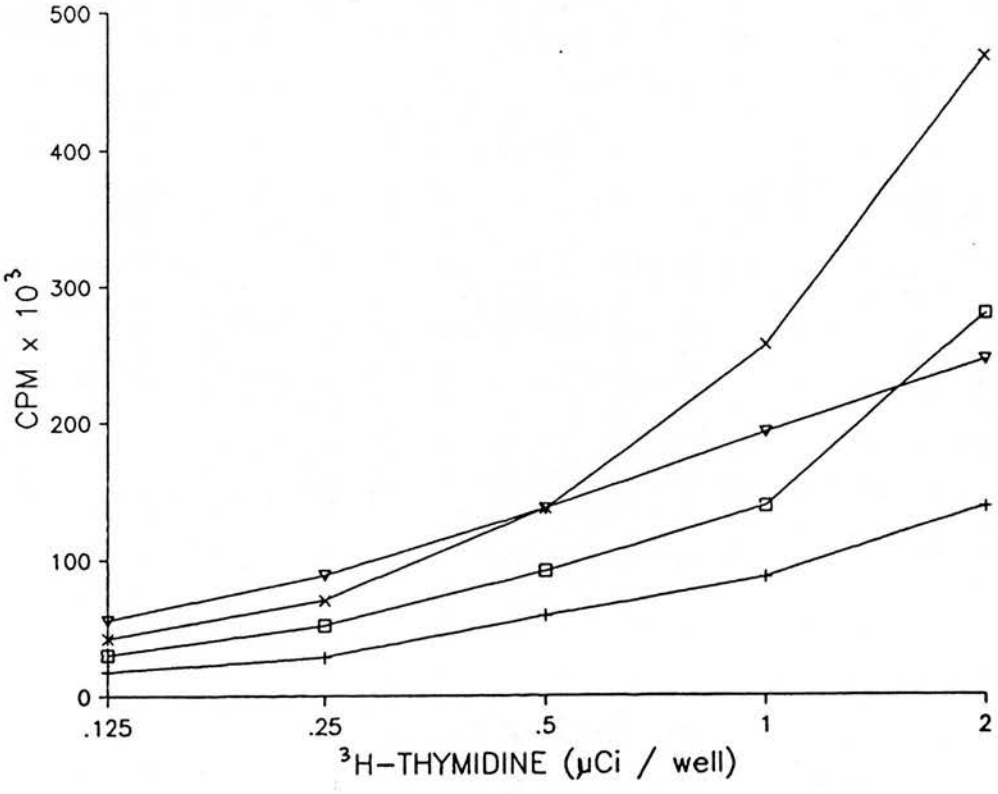

500
400
300
200
100
0
CPM x 10^3
.125
.25
.5
1
2
^{3}H–THYMIDINE (µCi / well)

FIGURE 2.8 Giemsa-stained cytospin of SMC from a *D.congolensis*-infected rat on day five of culture with an optimal concentration of cocci, showing predominantly blastoid cells.

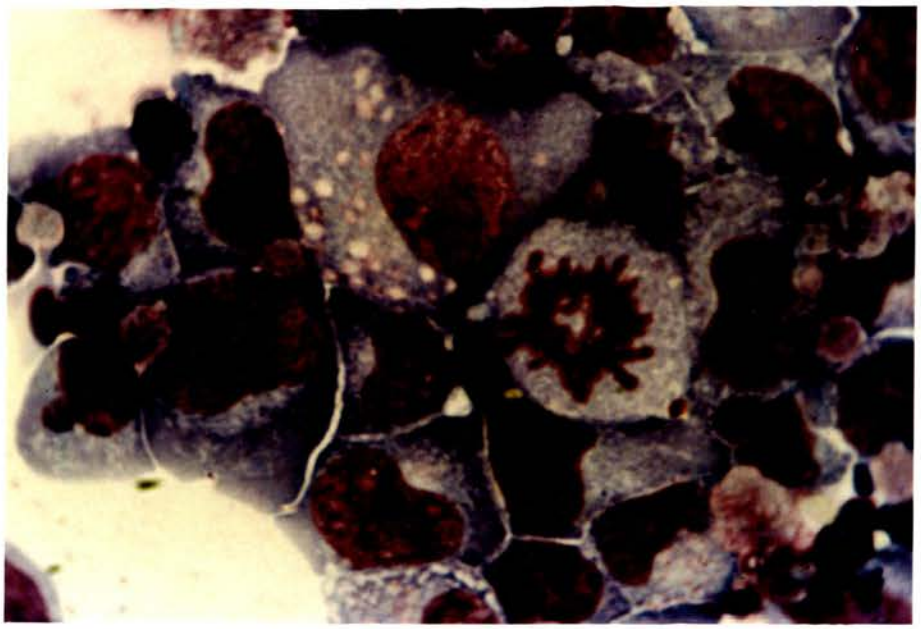
(x 1,060 magnification)

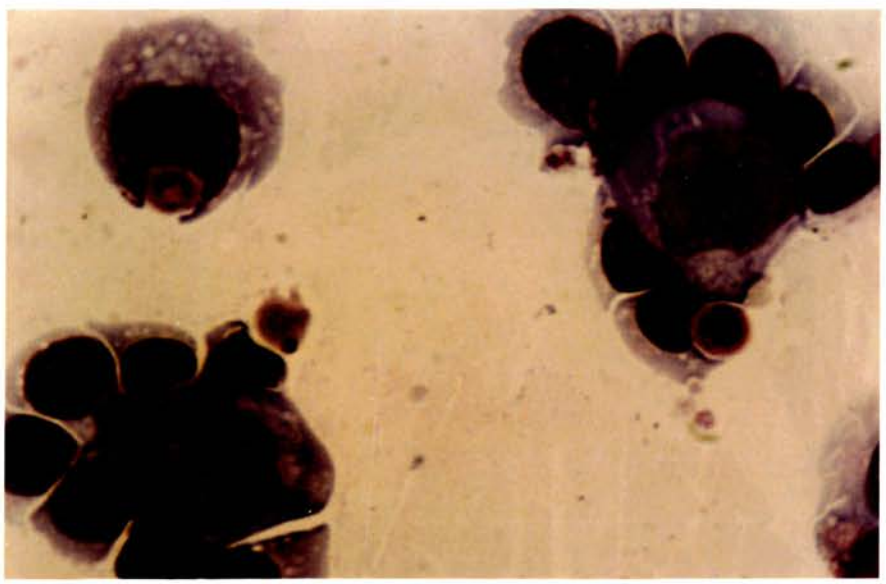
FIGURE 2.9 Giemsa-stained cytospin of SMC derived from the same rat as for figure 2.8 but cultured for five days in medium alone, showing mostly small lymphocytes.

(x 1,060 magnification)

FIGURE 2.10 Giemsa-stained cytospin of SMC derived from a naive rat and cultured for five days with an optimal concentration of *D.congolensis* cocci, showing few blastoid cells. Numerous cocci are visible in this field (arrow).

(x 1,060 magnification)





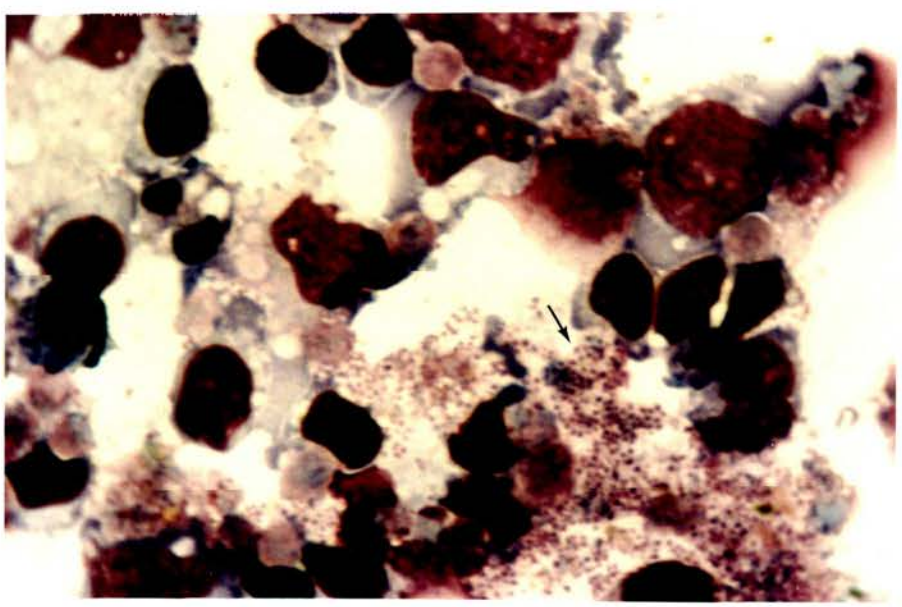


FIGURE 2.11 Stimulation indices for SMC cultured with various concentrations of *D.congolensis* cocci or with PPD at 20 $\mu g\ ml^{-1}$.

SMC from naive rat

SMC from *D.congolensis*-infected rat

SMC from PPD-sensitised rat

The inactivated cocci caused a significantly greater stimulation of SMC from infected rats than of SMC from naive rats at all concentrations tested (Mann-Whitney $U_s^s=7$, $P < 0.01$) and a significantly greater stimulation than that of SMC from PPD-sensitised rats over most of the concentration range (Mann-Whitney $U_s^s=7$, $P < 0.01$). Maximal stimulation was induced by a concentration of *D.congolensis* which resulted in a cocci:lymphocyte ratio of 8:1.

STIMULATION INDEX
0
2
4
6
8
10
12
3.9
4.9
5.9
6.9
7.9
PPD
log10 D.CONGOLENSIS COCCI / ml

CHAPTER THREE

IDENTIFICATION OF THE MONONUCLEAR CELL POPULATION RESPONDING TO *DERMATOPHILUS CONGOLENSIS IN VITRO*

CONTENTS

INTRODUCTION

Experimentally-induced infection by *Dermatophilus congolensis* primes rat mononuclear cells which will subsequently proliferate in response to *in vitro* stimulation by *D.congolensis* (Chapter 2). Whether the stimulation was a general effect on all mononuclear cell types, or whether a particular population was reacting was not known. The lymphocyte transformation test is a frequently-used indicator of cell-mediated immunity. However, lymphocyte transformation can also arise through stimulation of B-cell proliferation (Gery, Kruger and Spiesel 1972) by agents which cause an *in vivo* humoral response (Sjoberg 1971).

A range of anti-rat leucocyte monoclonal antibodies were used in an indirect fluorescent-antibody test to identify the immunophenotypes of the cells responding *in vitro* to *D.congolensis*. For comparison, the cell types in cultures derived from naive rats were also identified. The cell populations were examined both before and after five day's culture either in the presence, or the absence of an optimal concentration of *D.congolensis*.

MATERIALS AND METHODS

INFECTION WITH *D. CONGOLENSIS*

Dermatophilus congolensis was applied to the skin of inbred, male Wistar rats, approximately three to five months-old, following superficial skin scarification (p.52). The procedure was repeated twice at intervals of ten days, each infection being induced at a different site. Non-infected, control rats were subjected to the same regimen, except that the prepared skin surface was not inoculated with *D. congolensis*.

IDENTIFICATION OF IMMUNOPHENOTYPES OF SPLEEN MONONUCLEAR CELLS FROM NAIVE AND *D. CONGOLENSIS*-INFECTED RATS

SMC were isolated from rat spleens by the method described previously (p.44) on day ten after the third infection with *D. congolensis*, or day ten after the third scarification for controls. The SMC suspensions were each divided into two portions, one of which was used to set up cultures (p.92). The remaining SMC were suspended in Hank's basal salt solution containing one per cent bovine serum albumin (HBSS-BSA, Appendix 2) at a concentration of 2×10^6 cells ml^{-1}. The suspension was divided into 1 ml aliquots in Eppendorf tubes (Alpha-labs), which were then centrifuged at 800g for five minutes. The supernatant was removed and 25 µl of monoclonal antibody, at the appropriate dilution, was added to the pellet (p.89). The cells were resuspended in the monoclonal solution and incubated for 30 minutes at 4°C. Unbound antibody was removed with two washes in 1 ml HBSS-BSA (800g, 5 min.). The cells were then resuspended in 25 µl of fluorescein-isothiocyanate-conjugate (p.91) and incubated for 30 minutes at 4°C. Unbound conjugate was removed with a further two

washes as before. The cell pellet was then resuspended in 50 µl fixative (Appendix 2). After allowing the cells to fix for five minutes, a 10 µl aliquot was transferred to a clean glass slide and covered with a 22x22 mm coverslip. The edges were sealed with nail varnish and the cells observed under a fluorescence/phase contrast microscope with incident light (Leitz Laborlux K). For each field, the number of fluorescent (labelled) cells was counted under ultra-violet light (Figure 3.1) and the total number of cells recorded under phase-contrast (Figure 3.2). Over 200 cells were counted for each sample,

MONOCLONAL ANTIBODIES

Five monoclonals recognising determinants on the surface of various rat mononuclear cell populations were employed. All were obtained from Serotec and were in the form of ascites fluid. They were class IgG_1 and had been raised in mice. All monoclonals were diluted 1/10 with 0.5 per cent BSA in PBS and stored at -20°C in 100 µl aliquots (stock dilution). Repeated freeze-thawing was avoided.

A further monoclonal raised in the mouse was included as a negative control. This was 1C7 which was kindly donated by C.G.D. Brown (Shiels, Mc.Dougall, Tait, Brown 1986). 1C7 was a mouse IgG monoclonal recognising *Theileria* macroschizonts and was in the form of culture supernatant; it was used neat. All six monoclonals are detailed in table 3.1.

Table 3.1: Monoclonal antibodies used in the indirect fluorescent-antibody test

Monoclonal	Rat cell population possessing the recognised determinant
MRC OX-19	T lymphocytes and thymocytes
W3/25	T-helper lymphocytes. Also thymocytes and some macrophages
MRC OX-8	T-cytotoxic/suppressor lymphocytes. Also most natural killer cells and thymocytes
MRC OX-33	B-lymphocytes
ED1	Monocytes, macrophages and dendritic cells
1C7	None

Determination of Optimal Monoclonal Antibody Dilution

The recommended working dilution for all the anti-rat monoclonals was 1/100. However, since the degree of labelling varies with conditions of use, a representative monoclonal, MRC OX-19, was titrated out to determine the optimal working dilution.

Two-fold dilutions of MRC OX-19 were prepared in HBSS-BSA, from 1/50 to 1/800. These were tested on rat SMC, each with a range of conjugate dilutions. Although all the monoclonal dilutions tested gave positive results with the SMC, the fluorescence of the samples with OX-19 at 1/400 and 1/800 was slightly weaker than with the lower dilutions. Therefore, the working dilution chosen for these monoclonals was 1/200, being the

highest dilution giving strong fluorescence. The working dilutions, all prepared in HBSS-BSA, were stored at 4°C and used within a week.

FLUORESCEIN ISOTHIOCYANATE (FITC) CONJUGATE

The second antibody in the IFAT described was an affinity-purified sheep (heavy and light chain) anti-mouse IgG FITC conjugate (Sera-lab). The recommended working dilution for the (SAM) conjugate was 1/500. However, when titrated out to determine the optimum under the conditions of use, no labelling occurred at this dilution, nor at 1/250. At the same time it became apparent that at the lower dilutions (at which labelling of cells did occur), there was some non-specific binding, with about 20 per cent of SMC labelled regardless of whether they had been incubated with monoclonal or not. Thus, the sheep anti-mouse FITC conjugate itself had an affinity for rat cells. This problem was overcome by absorbing out the non-specific binding capacity of SAM prior to use (see below).

The pre-absorbed SAM conjugate was then titrated out against different dilutions of monoclonal to determine the optimal working dilution. Dilutions of 1/40, 1/60, 1/80 and 1/100 were tested and the optimum found to be 1/60, with weaker fluorescence at 1/80 and above.

The conjugate was stored at -20°C as a stock solution until ready for use when it was diluted in HBSS-BSA; thereafter, it was kept at 4°C, in the dark, and used within a week.

Absorption Out of Non-Specific Binding

Before the SAM conjugate was used in the IFAT, it was absorbed against rat SMC to remove the non-specific binding capacity. A whole rat spleen was dissociated and the cells separated on Ficoll gradients (p.44). About $1x10^8$ SMC were recovered and resuspended in 1/20 SAM conjugate (one

volume of cells to eight volumes of conjugate). The suspension was incubated for one hour at 4°C. The cells were then removed by centrifugation at 800g for ten minutes. The supernatant was recovered and filtered through a 0.22 μm Millex-GV unit. Before use, the absorbed conjugate was tested in an IFAT against rat SMC to check that all non-specific binding had been removed and that its activity had not been lost.

IDENTIFICATION OF IMMUNOPHENOTYPES OF SPLEEN MONONUCLEAR CELLS FOLLOWING *IN VITRO* STIMULATION BY *D.CONGOLENSIS*

SMC isolated from the same animals as those described above, were cultured by the same method as that for the lymphocyte transformation test (p.47), except that the culture vessels were 25 cm^{-2} flasks (Nunclon) instead of 96-well plates. The initial cell density was $2x10^{6}$ ml^{-1} and the total volume 10 to 15 ml. Four types of cultures were set up, SMC from infected rats or from naive rats, cultured either in complete medium alone, or with the addition of *D.congolensis* cocci.

D.congolensis was added to appropriate flasks at a pre-determined dilution to give 10^{5} to 10^{7} cocci ml^{-1}. Unfortunately, it was not possible to use exactly the same concentration of *D.congolensis* for each experiment, as a result of the method for determination of bacterial counts. Namely, serial dilutions of the bacterial suspension were plated out onto B/A and the number of colonies which had grown after 48 hours were counted (Appendix 1). Thus, the amount of *D.congolensis* added to the SMC cultures was based on an estimate, with the actual amount not known until two days later. However, variability in the *D.congolensis* concentration, between experiments, was minimised by using standard techniques for *D.congolensis* culture and harvest. Thus, the final culture

concentration was always at a level which was capable of causing significant stimulation, as assessed by the lymphocyte transformation test (Figure 2.11). Alternative methods of harvesting *D.congolensis* two days prior to use were unacceptable, since storage for this time, either at 4 or -20°C led to a decrease in the viable count.

The cultures were incubated at 37°C, under a 5 per cent carbon dioxide-95 per cent air atmosphere. On day three of culture, half the medium was removed, centrifuged at 300g for five minutes and the cell pellet resuspended in fresh medium. *D.congolensis* cocci were added, where appropriate, to maintain the concentration of stimulant and the cell suspension replaced in the culture flask.

On day five, the cultures were gently mixed to detach any adherent cells, then washed twice in PBS (300g, 5 min.). The cells were resuspended in 5ml HBSS-BSA and aliquots taken for Coulter counts. The suspensions were then diluted with HBSS-BSA to give $2x10^6$ cells ml^{-1}. The immunophenotype of the cells was determined as before (p.88).

RESULTS

IDENTIFICATION OF IMMUNOPHENOTYPES OF SPLEEN MONONUCLEAR CELLS FROM NAIVE AND *D. CONGOLENSIS*-INFECTED RATS

The mononuclear cell populations found in the spleens of rats ten days after the third and final infection, or for controls, ten days after the third scarification, are shown in table 3.2, which presents the median values derived from four experiments. There was essentially no difference between naive and infected rats, in the populations identified by the monoclonals.

Table 3.2: SMC phenotypes in freshly isolated spleen

Mc Ab	Specificity	Percentage labelled cells			
		Naive rat		Infected rat	
W3/25	Th	36	(33-42)	37	(26-43)
MRC OX-8	Tc/Ts	25	(21-27)	20	(20-27)
MRC OX-33	B	19	(13-23)	18	(10-20)
EDI	Mon/MØ	3	(2-4)	5	(3-7)
Total		83		80	

Values are medians from four experiments, in which over 200 cells were counted for each sample. The range is given in brackets. Mc Ab represents monoclonal antibody; these are detailed in table 3.1. The infected rat was tested on day 10 after the third infection with *D. congolensis* and the naive rat on the tenth day after the third scarification.

IDENTIFICATION OF IMMUNOPHENOTYPES OF SPLEEN MONONUCLEAR CELLS FOLLOWING *IN VITRO* STIMULATION BY *D.CONGOLENSIS*

SMC from the same sources as the cells identified in table 3.2, were cultured for five days, in the presence or absence of an optimal concentration of *D.congolensis*.

The cultures deriving from the naive rat did not display any dominant cell population, irrespective of whether *D.congolensis* was present or not (Figure 3.3, table 3.3). The composition remained similar to that on day zero; although the W3/25 (T-helper) population did show an apparant decrease by day five, the difference was not significant (Mann-Whitney $P > 0.05$).

In contrast, the cultures deriving from the infected rat showed a clear increase of the W3/25 population occurring only in the presence of *D.congolensis* cocci (Figure 3.4, table 3.3). This population was significantly greater than the W3/25 population in any of the other three types of culture (naive, plus/minus cocci, infected minus cocci) taken either singly (Mann-Whitney $U_4^4=0$, $P < 0.05$) or as a group (Mann-Whitney $U_{12}^4=3$, $P < 0.01$). With the increase in the percentage of W3/25, there was a concomitant decrease in chiefly the OX-33 (B-cell) population. There were no significant differences in the SMC populations between the unstimulated culture from infected rats and either of the cultures from naive rats (Mann-Whitney, $P > 0.05$).

The pan-T monoclonal, OX-19, consistently underestimated the T-cell population, as assessed by the two other anti-T cell monoclonals, W3/25 and MRC OX-8 (Table 3.4)

Table 3.3: Spleen mononuclear cell immunophenotypes in day five cultures

Monoclonal	Percentage labelled cells			
	Naive minus D.congolensis	Naive plus *D.congolensis*	Infected minus *D.congolensis*	Infected plus *D.congolensis*
W3/25 (Th)	28 (14-35)	27 (19-39)	30 (22-38)	56 (52-64)
MRC OX-8 (Tc/Ts)	28 (9-38)	26 (20-29)	18 (13-27)	16 (11-19)
MRC OX-33 (B)	15 (12-22)	18 (16-24)	16 (11-22)	6 (5-13)
ED1 (Mon/MØ)	7 (5-8)	3 (3-4)	8 (6-8)	4 (3-5)
Total	78	74	72	82

Values are medians from four experiments in which over 200 cells were counted for each sample. The range is given in brackets. SMC were cultured in the presence or absence of an optimal concentration of *D.congolensis*. The cells were derived from the same animals as in table 3.2.

Table 3.4: Labelling of SMC by the monoclonal OX-19 compared with W3/25 and MRC OX-8

	Percentage labelled cells		
	OX-19		Sum W3/25 and MRC OX-8
i)Day 0			
Naive	56	(56-61)	61
Infected	47	(44-57)	57
ii)Day 5			
Naive minus *D.congolensis*	34	(22-51)	56
Naive plus *D.congolensis*	36	(18-42)	53
Infected minus *D.congolensis*	42	(28-50)	48
Infected plus *D.congolensis*	36	(28-52)	72

Values are medians from the same eight experiments reported above (Tables 3.2 and 3.3) for i) freshly isolated SMC and ii) day five cultures, plus or minus an optimal concentration of *D.congolensis* cocci. The range is given in brackets.

DISCUSSION

Earlier, it was shown that *in vitro* exposure of SMC from infected rats to *D.congolensis* caused a stimulation of cell proliferation (Figure 2.11). Immunophenotyping of SMC, derived from infected rats and cultured under the conditions present during the lymphocyte transformation test, revealed a large increase in the W3/25 population. This population had increased to form 56 per cent of the total, a figure which was significantly higher than that in unstimulated SMC from infected rats, or SMC from naive rats, whether cultured with *D.congolensis* or not (Table 3.3).

W3/25 positive cells are T-lymphocytes characterised by their helper function and lack of suppressor activity (White, Mason, Williams, Galfre and Milstein 1978) and by their release of interleukin-2 when stimulated (Cantrell, Robins and Baldwin 1982). In addition, Barclay (1981) reported labelling of some macrophages with W3/25, although in spleen the labelling was diffuse and difficult to correlate with recognisable cells.

The expansion of the W3/25 population appeared to be the only significant deviation from the normal cell populations. However, absolute cell numbers of each mononuclear cell subset were not determined in this study, which was designed to identify any expanding subsets for each type of SMC culture. Thus, it is not possible, for example, to determine whether an actual decrease of MRC OX-33 (B-cells) occurs when SMC from infected rats are cultured with *D.congolensis*, or whether the apparent decrease is merely a reflection of the increased percentage of cells which are W3/25 positive. It is interesting to note that although *D.congolensis* cocci do cause some non-specific stimulation of SMC in the

lymphocyte transformation test (Figure 2.11), no predominant immunophenotype was identified in these cultures (Table 3.3), thus the stimulation of non-sensitised SMC appears to be truly non-specific.

The monoclonal antibody MRC OX-19 proved to be disappointing as a pan T-cell marker. When the percentage of SMC labelled with OX-19 was compared with the sum total of those labelled with W3/25 or OX-8, the former value was always lower than the latter (Table 3.4). Although the value was lower the ranges did overlap, except for SMC from infected rats cultured with *D.congolensis*; here, only 36 per cent of cells were labelled with OX-19 compared with the significantly higher 72 per cent labelled with W3/25 or OX-8 (Mann-Whitney $U_4^4=0$, $P < 0.05$).

It is possible that OX-8 and W3/25 together overestimate the T-cell population, rather than OX-19 underestimating it. This could arise if some of the cells were expressing both the antigens recognised by W3/25 and OX-8. However, although a recognised phenomenon, only about two per cent of T-cells normally express both markers (Blue, Daley, Levine and Schlossman, cited by Jefferies 1988). Also if this were true, it would mean that only 41 per cent of cells were of an identifiable immunophenotype in the stimulated SMC from infected rat cultures (derived by taking the median value of the sum of those cells labelled by OX-19, OX-33 and ED1). In view of this, together with the fact that a clear and strong response is occurring in these cells, the most likely explanation for the discrepancy is an underestimate of the T-cell population by OX-19. Scolley (1986) pointed out that pan-T markers have been difficult to find and that a combination of markers is just as, or more, useful. It is also noteworthy that certain antigens are lost on activation of the cells, for example, the determinant recognised by the monoclonal OKT17 is present on all circulating mouse T-cells, but is lost from a T subset

after activation with pokeweed mitogen (Thomas, Rogozinski, Irigoyen, Shen et al 1982).

Determination of the immunophenotypes present in freshly-isolated SMC revealed a similar distribution of populations for cells from naive or day ten *D.congolensis*-infection rats (Table 3.2). The SMC consisted of about 36 per cent W3/25 positive (T-helper) and about 23 per cent MRC OX-8 positive (T-cytotoxic/suppressor) cells. These values agree quite well with those of Brideau, Carter, McMaster, Mason and Williams (1980) who found that 29 per cent and 27 per cent of rat spleen lymphocytes labelled with W3/25 and MRC OX-8 respectively. The remaining cells (Table 3.2) consisted of about 18 per cent B-lymphocytes, which were labelled with MRC OX-33, and around 4 per cent monocytes or macrophages, which were labelled with ED1.

It is, perhaps, not surprising that rats, ten days after a third infection, possess similar SMC populations to naive rats. *D.congolensis* infections remain localised in the skin and it is the infection site itself, or its draining lymph node, where *in situ* changes in mononuclear cell populations are most likely to be seen. However, it is possible that if the spleen cell populations were examined over the duration of the infection, rather than at one time point, deviation from the normal values would be seen; for example soon after a repeat infection. Nevertheless, a proportion of the SMC from these infected rats must be primed, as evidenced by the specific response to *D.congolensis* in the lymphocyte transformation test (Figure 2.11), although the actual numbers of these cells may be small, with clonal expansion only occurring in the presence of further stimulation, either *in vivo* or *in vitro*.

The total percentage of cells labelled by the battery of monoclonal antibodies ranged from 80-83 per cent for freshly isolated SMC and 72-82

per cent for SMC day five cultures (Tables 3.2 and 3.3). There are several factors which could account for the unlabelled 17-28 per cent of cells. Firstly, a significant proportion of mononuclear cells lack both T-cell, B-cell and monocyte/macrophage surface markers and functional characteristics. These are the null cells which constitute three to fourteen per cent of mouse SMC (Stobo, Rosenthal and Paul 1973). Null cells are a heterogenous group; some are natural suppressor cells (May, Slavin and Vitetta 1983) but the majority, at least in human blood, are natural killer cells (Ligthart, Van Vlokhoven, Schuit and Hijmans 1986). The monoclonal antibody MRC OX-8 used in this study, should label most natural killer cells (Cantrell, Robins, Brooks and Baldwin 1982), so only some of the unlabelled SMC can be accounted for by virtue of their null cell phenotype.

The spleen is one of the sites where lymphocytopoiesis occurs (Pabst, Licence and Binns 1983); in mouse spleen about six per cent of the cells are haemopoietic (Twomey and Kouttab 1981). Thus, a proportion of the spleen cells may be immature mononuclear cells which lack the markers present on differentiated cells, some of which will be the determinants recognised by the monoclonals.

A further consideration is that any non-viable cells may well have lost the surface molecules to which the monoclonals bind. However, non-viable cells are easily distinguishable since their membrane is permeable to the conjugate such that the whole cell fluoresces brightly (Johnstone and Thorpe 1982) and these cells were not included in the cell counts. Finally, one cannot discount the possibility that the monoclonal antibodies used do not label all of the cells they are described as recoginising.

The percentage of cultured cells labelled was generally slightly lower

than that of SMC on the day of isolation from the animal. This may be due to alterations in some structural characteristics, brought about by the *in vitro* environment, such as loss of some surface antigens in culture (Jessen and Mirsky 1983).

In summary, W3/25 positive cells proliferate *in vitro* in response to stimulation by *D.congolensis*, following priming of the donor animal by experimentally-induced infection. *D.congolensis* may cause stimulation of the same cells *in vivo*, posing the question of what effect these activated cells might have on the host response to infection. Possible implications of a predominantly T-helper response to *D.congolensis* will be discussed in later chapters.

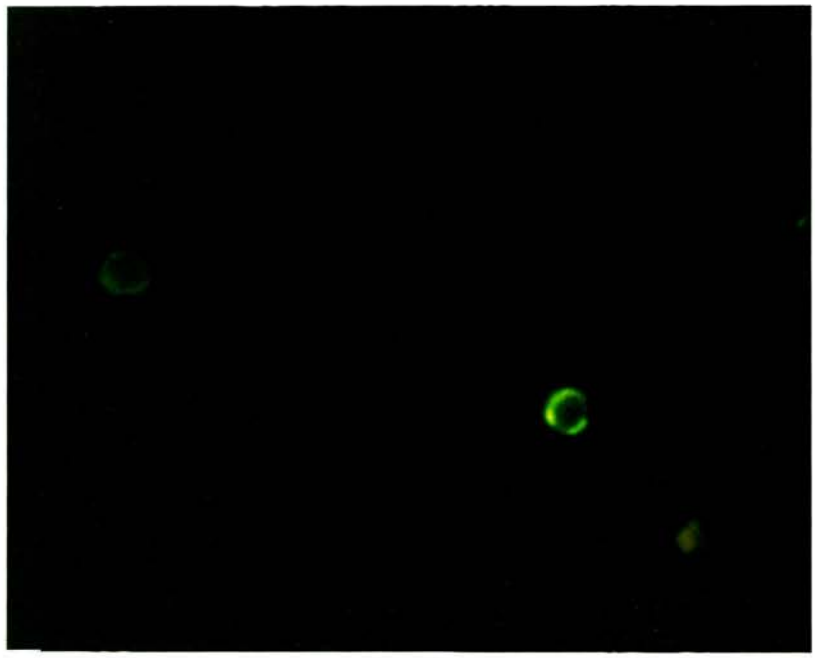
One of the main properties of activated T-helper cells is the release of a wide range of lymphokines; these are essential effector molecules in the initiation and maintainance of an immune response (Alm 1987). Whether lymphokines were being produced by sensitised SMC in response to *in vitro* stimulation with *D.congolensis* forms the basis of the following chapter.

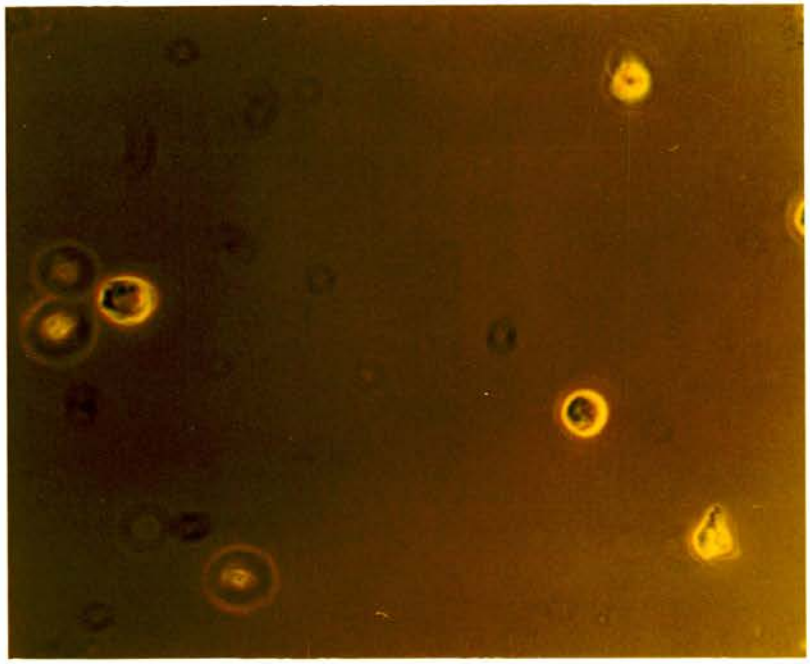
FIGURE 3.1 Fluorescent cultured mononuclear cell labelled with the monoclonal antibody W3/25 and visible under ultra-violet light. The weakly-staining cell is read as negative.

(x 425 magnification)

FIGURE 3.2 The same field shown in figure 3.1 under phase contrast.

(x 425 magnification)





FIGURES 3.3 and 3.4 Immunophenotypes of SMC from infected or naive rats on day five of culture either in the presence or the absence of an optimal concentration of *D.congolensis* cocci.

Percentage cells labelled by the monoclonal antibodies:

OX-33	B-lymphocyte marker
W3/25	T-helper marker
OX-8	T-cytotoxic/suppressor marker
ED1	Monocyte and macrophage marker

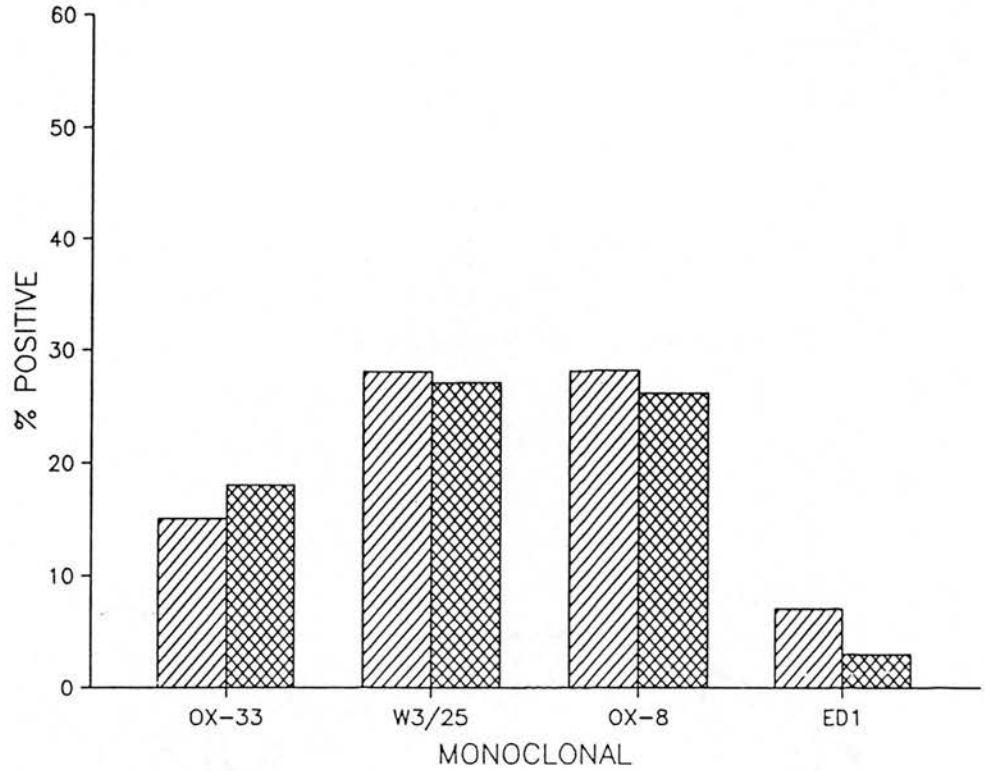
Striped bars represent SMC cultured in medium alone

Hatched bars represent SMC cultured with an optimal concentration of *D.congolensis* cocci.

Values are medians from four experiments

Figure 3.3 SMC from naive rats.
The presence of *D.congolensis* cocci had no significant effect on the distribution of the immunophenotype populations.

Figure 3.4 SMC from *D.congolensis*-infected rats.
A marked expansion of the W3/25-positive population of cells occurred in the presence of cocci. This population was significantly larger than the W3/25 population in any of the other types of culture (Mann-Whitney $U_4^4=0$, $P < 0.05$). No such response occurred in the absence of cocci.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50
40
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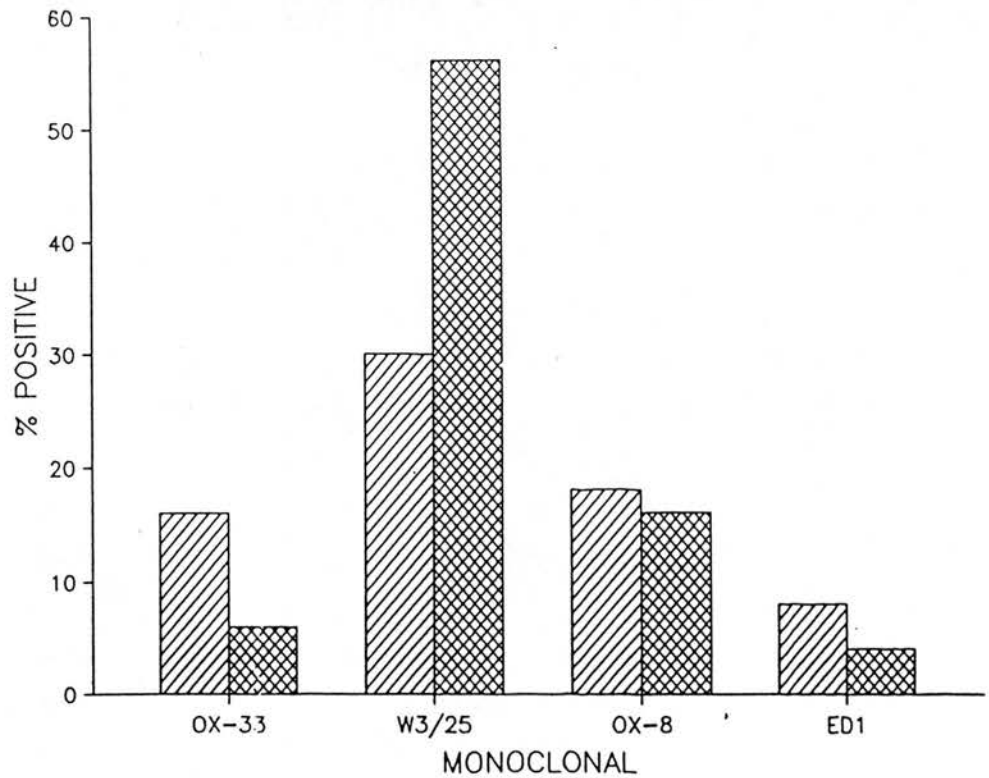
% POSITIVE
OX-33
W3/25
OX-8
ED1
MONOCLONAL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OX-33
W3/25
OX-8
ED1
MONOCLONAL

CHAPTER FOUR

PRODUCTION OF LYMPHOKINE-CONTAINING MONONUCLEAR CELL CULTURE SUPERNATANTS

CONTENTS

INTRODUCTION

In the previous chapters, *Dermatophilus congolensis* was shown to induce proliferation of cultured mononuclear cells isolated from the spleens of *D.congolensis*-infected rats. This proliferation was accompanied by expansion of the W3/25 positive (T-helper) population. The next step taken was to determine whether the *in vitro* stimulation of SMC by *D.congolensis* caused the release of lymphokines from these cells.

The term lymphokine applies to a wide range of non-immunoglobulin, polypeptide effector molecules, synthesised and released by stimulated lymphocytes; monocyte-derived factors are often included in this category, although sometimes defined separately as monokines (Pick, Cohen and Oppenheim 1979). Lymphokines are essential components in virtually every part of the immune response. Their activities include growth and differentiation promotion, growth inhibition, enhancement or suppression, activation of phagoctyic cells and attraction and localisation of inflammatory cells (*Ibid.*).

The first lymphokine to be described was macrophage migration-inhibition factor (MMIF). In an *in vitro* assay developed by George and Vaughan (1962), peritoneal exudate cells, derived from hypersensitive guinea-pigs, exhibited a marked reduction in migration in the presence of specific antigen. Whereas, cells from non-sensitised animals showed no inhibition of migration. Cells from animals manifesting a humoral response only to an antigen, did not exhibit migration inhibition in the presence of the antigen (David, Al-Askari, Lawrence and Thomas 1964). Bloom and Bennett (1966) demonstrated that the migration inhibition was due to a soluble factor (MMIF) which was produced by antigen-stimulated lymphocytes; whilst the macrophage component provided the indicator

population.

The macrophage migration "factor", which is stable at 37°C and to lyophilisation, was later shown to constitute an heterogeneous group of molecules, with molecular weights ranging from 12,000 to 82,000 daltons (Yoshida 1979). The exact mechanism by which MMIF causes inhibition of macrophage migration is unclear, but it is likely that MMIF binds to specific receptors on the cell surface causing generation of an intracellular second messenger. This then brings about changes in the cytoskeleton, a structure which plays a central role in macrophage cell movement (Pick 1979). Macrophage migration-inhibition factor has been defined by its *in vitro* action; the results from direct MMIF tests do, however, show a reasonable correlation with the *in vivo* cell-mediated immune status of the animal (Reviewed by Turk 1975).

The culture supernatants of *D.congolensis*-stimulated SMC were assayed for the presence of macrophage migration inhibition activity in an indirect test based on the original, direct one described by George and Vaughan (1962). For comparison, SMC culture supernatants, derived from both *D.congolensis*-infected and naive rats were assayed for MMIF activity.

MATERIALS AND METHODS

ANIMALS

Dermatophilus congolensis was applied to the skin of inbred, male Wistar rats, approximately three to five months-old, following superficial skin scarification (p.52). The procedure was repeated twice at intervals of ten days, each infection being induced at a different site. Non-infected, control rats were subjected to the same regimen, except that the prepared skin surface was not inoculated with *D.congolensis*.

PREPARATION OF MONONUCLEAR CELL CULTURE SUPERNATANTS

Spleen mononuclear cells were isolated as described previously, ten days after commencement of the third and final infection (p.44), or, for controls, ten days after the final scarification. The supernatants were prepared by a method, based on that described by Korszun, Wilton and Johnson (1981) for PPD-stimulated guinea-pig spleen cells.

The cells were suspended in complete RPMI-1640 medium (Appendix 1) and the yield was then split into two halves in 25 cm^{-2} culture flasks. To one flask, *D.congolensis* cocci, harvested and diluted in complete medium, as before were added to give a final concentration of 10^{6}-10^{7} cocci ml^{-1} (see p.92).

The second SMC culture, to which *D.congolensis* was not added, acted as a non-stimulated control. Further complete medium was added to both flasks to achieve a cell concentration of $5x10^{6}$ SMC ml^{-1} and a culture volume which was the same in each. The flasks were incubated at 37°C, under a 5 per cent carbon dioxide-95 per cent air atmosphere for 24 hours. The remaining *D.congolensis* suspension in complete medium was

incubated under identical conditions.

At the end of the incubation period, the cultures were chilled to 4°C, then an equivalent amount of the *D.congolensis* suspension was added to the unstimulated culture, such that the control supernatant would contain cocci, and any released factors, at the same concentration, as the stimulated SMC culture supernatant. The cells were pelleted by centrifugation at 700g, for ten minutes at 4°C. The supernatants were harvested and filtered through a 0.22 µm low protein absorption filter unit. Half of each supernatant was then stored at -20°C, whilst the remainder was dialysed against 100 volumes incomplete medium for 24 hours, at 4°C, with one change of medium. The dialysed supernatants were filter sterilised through low protein absorption filters and stored at -20°C.

For use, the supernatants were diluted with the appropriate medium, taking into account the fact that the control supernatant had already been diluted by the addition of *D.congolensis* suspension at the end of the incubation period.

MACROPHAGE MIGRATION INHIBITION TEST

The dialysed SMC culture supernatants, prepared as above, were assayed for macrophage migration inhibition activity, using an indirect test. This was adapted from the direct test described by Higgins (1983) which, in turn, was based on the original assay of George and Vaughan (1962). Aseptic techniques were used throughout.

Normal adult, male Wistar rats were used as the source of macrophages for the assay. Forty-eight hours prior to harvesting the cells, the rats were injected intra-peritoneally with 4 ml of an emulsion consisting of equal volumes of Freund's incomplete adjuvant and Hank's balanced salt

solution (HBSS). This procedure was carried out to induce a peritonitis, such that high yields of macrophages would be obtained.

The rats were exsanguinated, under a general ether anaesthetic, to reduce erythrocyte contamination of the peritoneal exudate. The abdominal skin was reflected and 10 ml of HBSS containing 5 units ml^{-1} heparin (Appendix 3) injected into the peritoneal cavity; gentle massaging of the abdomen ensured that the solution was evenly distributed. The peritoneal cavity was then opened and the mixture of cellular exudate, adjuvant and HBSS, totalling a volume of 7-12 ml, was collected into a sterile syringe.

To remove the adjuvant from the suspension, the syringe was left to stand vertically for 40-50 minutes, after which, the cells were dispensed from the bottom of the syringe, leaving most of the adjuvant at the top. The suspension was washed twice in HBSS/heparin (200g, 5 min. at room temperature) and then twice in Eagle's Minimal Essential Medium containing nine per cent FCS (Appendix 3). After each wash, any remaining traces of Freund's adjuvant, on the supernatant surface, were removed with a pasteur pipette.

The cell concentration of the suspension was determined using a haemocytometer, after dilution with 0.2 per cent trypan blue (p.46) and was then adjusted to $5x10^7$ ml^{-1} with complete medium. Capillary tubes (Hawksley) were filled with the cell suspension, which was kept well mixed, and one end of each tube was sealed with a proprietary clay (Hawksley). The tubes were centrifuged at 200g for five minutes at room temperature and then broken at the interface between cell pellet and fluid, after first scoring the glass with a diamond pen. The resultant stubs were placed in wells of migration plates (Sterilin) with the cut end projecting towards the centre of the well and the sealed end held in

place at the rim with sterile silicone grease. To each well, 0.4 ml of appropriate supernatant, either *D.congolensis*-stimulated or control, diluted in complete Eagle's medium was added and the chamber sealed with a coverslip, after the well rims had been smeared with silicone grease. The plates were incubated at 37°C for 18 hours.

The degree of cell migration for each sample, was determined by directly measuring the distance the population of cells had travelled. This was carried out by incorporating a micrometer scale in the eyepiece of an inverted microscope. For each sample, the position of the micrometer scale was adjusted such that it was parallel to the length of the capillary tube and the distance between the leading edge of the main body of migrating cells and the cut end of the tube was measured.

Although care was taken in the filling of the wells and application of the coverslips, air bubbles formed in some of the chambers. Where this occurred the migration of the cells was often affected and the result had to be discarded. Due to this inevitable loss of some of the samples and to variable yields of cells, the number of replicates varied from 5 to 13 for each supernatant. Only dialysed culture supernatants were tested for migration-inhibition activity.

RESULTS

MACROPHAGE MIGRATION INHIBITION TEST

The cells harvested from the peritoneal exudate consisted of about 50 per cent macrophages, 15 per cent lymphocytes, 20 per cent PMN, 10 per cent eosinophils and 5 per cent erythrocytes (Figure 4.1).

The supernatants which were assayed for macrophage migration inhibition activity were from SMC cultured either in the presence or absence of an optimal concentration of *Dermatophilus congolensis*. Two cell types were used for producing culture supernatants: SMC derived from rats which had been infected with *D.congolensis* and SMC from naive rats.

The results were expressed as the migration index, which was calculated as follows:

$$\text{Migration Index} = (Mdt / Mdc). 100 \qquad (2)$$

Where Mdt is the median distance migrated by cells in the presence of test (*D.congolensis*-stimulated) supernatant and Mdc is the median distance migrated by cells in the presence of control (unstimulated) supernatant

Whether or not significant inhibition of migration had occurred was determined with the Mann-Whitney test. In the presence of supernatants derived from infected rats, significant inhibition of migration occurred with the test, as compared with the control supernatant in all five experiments. The median migration distances were reduced to between 43 and 78 per cent of those in the presence of control supernatant (Table 4.1). Although the greatest inhibition occurred with the lowest dilution

of supernatant, the degree of inhibition was not totally dose-dependent. This is, however, likely to be, at least partly, a reflection of the variability between experiments.

Figure 4.2 shows peritoneal exudate cells, after incubation for 18 hours with a control, unstimulated, supernatant, derived from an infected rat. Figure 4.3 shows cells harvested from the same rat, after 18 hours of incubation with a *D.congolensis*-stimulated supernatant, derived from the same infected rat. The area of cell migration from the capillary tube is much smaller in the presence of the test supernatant than in that of the control.

Culture supernatants derived from naive rats, did not cause significant inhibition of peritoneal exudate cell migration (Table 4.2). Although the migration index was as low as 77 per cent with the 1/2 dilution of supernatant, in none of the experiments did the test supernatant cause significant inhibition of migration when compared with the control supernatant.

In initial experiments, the distance which the peritoneal exudate cells migrated when incubated with *D.congolensis*-stimulated culture supernatant was compared with that migrated in culture medium alone, as well as that in the presence of a control culture supernatant. Both the test and control supernatants caused inhibition of migration compared with medium alone. But the inhibition caused by the test supernatant was greater than that caused by the control. In later experiments, including all those reported here, the migration in medium alone was not determined, so as to allow a larger number of replicates to be set up for the two factors of interest, namely *D.congolensis*-stimulated and control supernatants.

Table 4.1: Migration distances in the presence of SMC culture supernatants derived from *D.congolensis* infected rats.

Expt.	Dilution of supernatant	Migration with test supernatant (units)	Migration with control supernatant (units)	Migration Index (%)	Level of significance
1	1/2	30 (20-80)	70 (35-90)	43	$P < 0.05$
2	1/10	35 (25-65)	55 (50-80)	64	$P < 0.05$
3	1/10	55 (20-70)	70 (50-80)	78	$P < 0.05$
4	1/10	55 (40-70)	80 (55-105)	69	$P < 0.05$
5	1/20	50 (50-60)	90 (80-100)	56	$P < 0.01$

The values are medians of 5 to 13 replicates, rounded to the nearest 5 units. Those in brackets denote the range. Significance was determined by the Mann-Whitney test. One unit is equivalent to 20 µm. The supernatants in experiments one and five had been stored lyophilised and were reconstituted in the appropriate volume of medium, the others had been stored and diluted as described in the method. The migration distances from which the medians are calculated are presented in appendix three.

Table 4.2: Migration distances in the presence of SMC culture supernatants derived from naive rats.

Expt.	Dilution of supernatant	Migration with test supernatant (units)	Migration with control supernatant (units)	Migration Index (%)	Level of significance
1	1/2	50 (30-70)	65 (25-70)	77	NS
2	1/10	72 (60-85)	72 (50-80)	100	NS
3	1/10	50 (20-80)	58 (35-80)	86	NS

The values are medians of 7 to 10 replicates, rounded to the nearest 5 units. Those in brackets denote the range. NS represents not significant (Mann-Whitney, $P > 0.05$). One unit is equivalent to 20 μm. The supernatant in experiment one had been stored lyophilised, the others had been stored and diluted as described in the method. The migration distances from which the medians are calculated are presented in appendix three.

DISCUSSION

When lymphocytes are stimulated *in vitro* by mitogens, most lymphokines begin to appear in the culture supernatant about four hours later, with peak levels being reached within 12-24 hours; synthesis then stops (Kappler and Marrack 1986). Likewise, peak lymphokine levels in antigen-stimulated T-cell cultures occur within 24 hours (*Ibid.*). Thus, the supernatants in this study were harvested 24 hours after initiation of culture.

Culture supernatants from *D.congolensis*-stimulated SMC, isolated from infected rats, caused significant inhibition of peritoneal exudate cell migration (Table 4.1). The supernatants were not characterised, so it is not possible to identify the active factor as MMIF itself, although, as already stated, MMIF activity is not confined to one molecule (Pick 1979). Furthermore, other factors released by lymphocytes, which inhibit PMN motility may be present (Pick 1979) and may have contributed to the inhibition of the peritoneal exudate which contained significant numbers of PMN.

Only dialysed culture supernatants were assayed for the presence of MMIF. This was because MMIF is non-dialysable ie. has a molecular weight of over 10,000 daltons (Yoshida 1979), which is the cut-off point for dialysis tubing (Johnstone and Thorpe 1982). Also, non-specific factors can interfere with macrophage migration (Ling and Kay 1975) and some of these can be removed by dialysis.

The most common method of determining extent of cell migration, in a macrophage migration inhibition assay, is to project the images of the migration areas onto a screen, trace their outline onto paper and then cut out and weigh the pieces. The weight of the paper is then taken as

being directly proportional to the degree of migration. Watanuki and Haga (1977) used the more simple method of directly measuring the distance of migration using an eyepiece micrometer. They demonstrated that this method gave results which were as statistically sound as those for the more traditional method despite the fact that the migration distances were not perfect spheres. For this reason and for convenience, the method of direct measurement was used in this study.

The analysis of results from macrophage migration inhibition assays deserves some consideration. It has been customary to consider inhibition has occurred when the migration index is less than, or equal to the arbitary value of 80 per cent used by Bloom and Bennet (1966). A more valid approach, however, is to calculate whether the difference in migration distances between test and control samples is statistically significant.

In this study the migration of cells in the presence of test supernatant was compared with that in the presence of control supernatant. Morris, Stevens and Hebert (1976) claimed that a further set of controls should be included, that of culture medium with and without added antigen, ie. in the absence of culture supernatants. These controls are largely superfluous when, as is the case in this study, any effects of the antigen itself are allowed for by reconstituting the control supernatant with antigen before assaying for migration inhibition. Furthermore, the inclusion of these extra controls decreases the number of replicates per sample possible when the source of macrophages is limited, as it is here. A wide scatter of data within replicates is inherent in the migration inhibition assay, so it is important to include as many replicates as possible (Hudson and Hay 1976).

Macrophage migration inhibition factor is synthesised and released by lymphocytes which have received either a specific antigen stimulus (Bennett and Bloom 1967) or a non-specific mitogen stimulus (Pick, Brostoff, Krejci and Turk 1970). It has already been shown that *Dermatophilus congolensis* causes some non-specific stimulation of SMC, as well as the more powerful stimulation of pre-sensitised cells, as demonstrated by the results from the lymphocyte transformation test (Figure 2.11). In the light of this non-specific stimulation of SMC from naive rats, it might be assumed that these cells, when stimulated with *D.congolensis*, would release MMIF. The results presented here suggest this is not the case (Table 4.2). The lack of migration inhibition activity by the naive rat supernatants can be explained by an examination of the cell phenotypes present in the cultures. Whereas, in SMC cultures from infected rats, the T-helper population expanded in response to *in vitro* stimulation with *D.congolensis*, no such response occurred in SMC cultures from naive rats (Table 3.3). Thus, the cells which are responsible for MMIF production, (Roitt *et al* 1985) were activated and proliferating in cultures from infected rats, but not in cultures from naive rats. MMIF activity may, nevertheless, be present in the test supernatants from naive rats, but at levels too low to cause significant inhibition of macrophage migration.

The population of cells which produced the migration inhibition activity is probably the same as that responding in the lymphocyte transformation test, this being the W3/25 positive (T-helper) population identified in the indirect fluorescent antibody test. The culture supernatants of activated lymphocytes contain many different lymphokine activities (Kappler and Marrack 1986). It has been shown that the supernatants of *D.congolensis*-stimulated SMC possess one type of activity

associated with a known lymphokine, MMIF. Chapter six details the assessment of the supernatants for the presence of other lymphokines which might affect epidermal cell growth. The development of an epidermal culture system, suitable for this purpose, is described next, in chapter five.

FIGURE 4.1 Peritoneal exudate cells from a rat 48 hours after intra-peritoneal injection of Freund's incomplete adjuvant.

Giemsa-stained cytospin showing:

MØ	Macrophage
L	Lymphocyte
P	Polymorphonuclear leucocyte
Eo	Eosinophil
Er	Erythrocyte

(x 425 magnification)

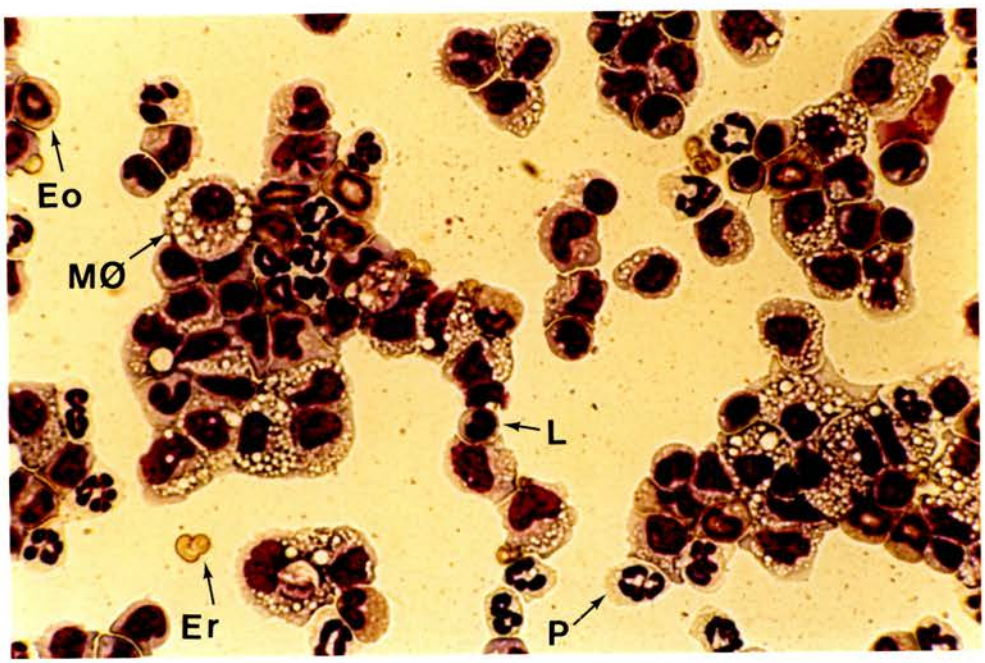
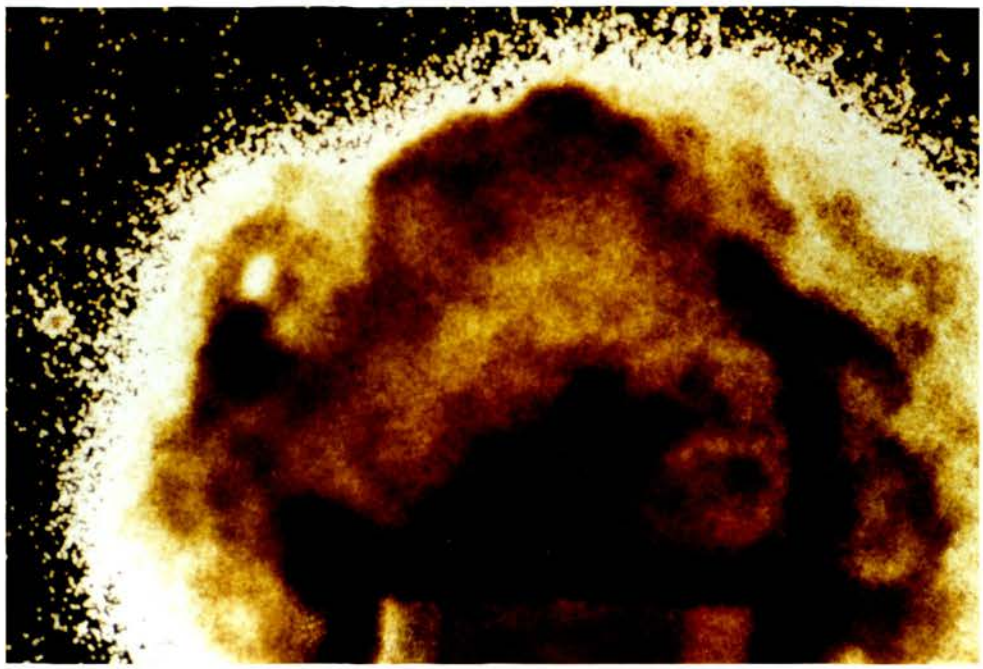
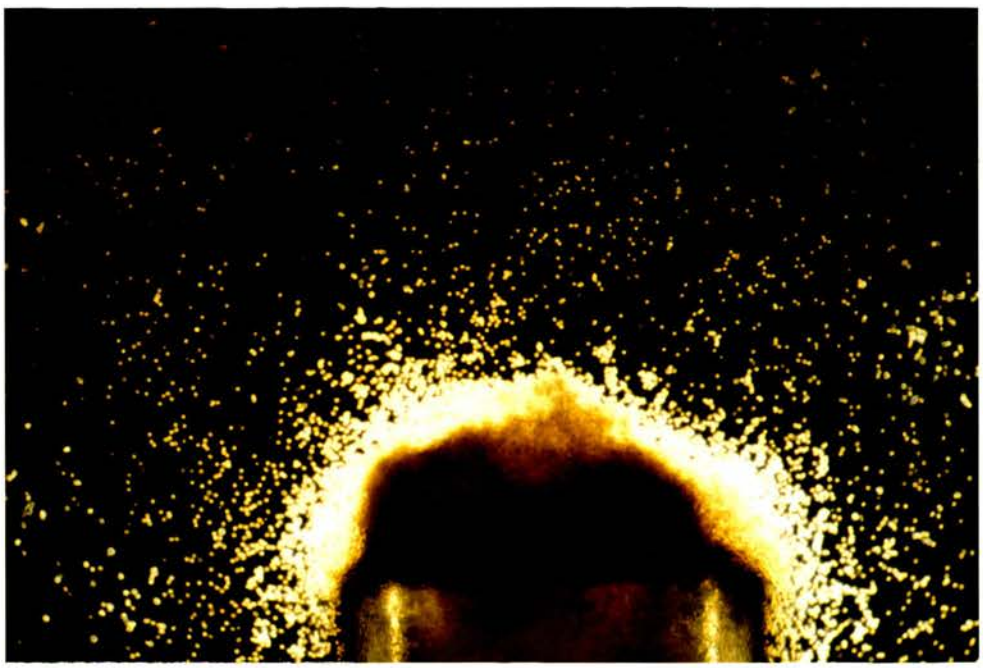

Eo
MØ
L
Er
P

FIGURE 4.2 Migration of peritoneal exudate cells from a capillary tube in the presence of control supernatant. Phase contrast.
(x 42 magnification)

FIGURE 4.3 Inhibition of the migration of peritoneal exudate cells from a capillary tube in the presence of supernatant derived from *D.congolensis*-stimulated mononuclear cells from infected rats. Phase contrast.
(x 42 magnification)





CHAPTER FIVE

EPIDERMAL CELL CULTURE

CONTENTS

CONTENTS (cont.)

INTRODUCTION

The aim of the work described in this chapter was to develop an epidermal cell culture system with proliferation and differentiation characteristics similar to those of the epidermis *in vivo*.

Many different methods for culturing epidermal cells have been employed, with varying degrees of success in terms of how representative the cultures were of the *in situ* epidermis and have been reviewed by Holbrook and Hennings (1983). The most widely used culture system is that of Rheinwald and Green (1975) who grew epidermal cells on an irradiated, mouse 3T3, fibroblast feeder layer. Human epidermal cultures, established by this method, have been used successfully as skin grafts (O'Connor, Mulliken, Banks-Schlegel, Kehinde and Green 1981). The cultures were multilayered and possessed many of the morphological characteristics of normal epidermis but terminal differentiation, with formation of a *stratum corneum* only occurred once the cultures had been grafted onto the recipient patient (Faure, Mauduit, Schmitt, Kanitakis *et al.* 1987).

One drawback of this type of culture system is that, in studies of the effect of various factors on the epidermal cells, it is impossible to distinguish whether the effect is a direct one or an indirect one mediated by the feeder layer cells. The problem may be overcome with a culture system containing epidermal cells only. Such cultures have been described for animal, as well as human, cells (Holbrook and Hennings 1983).

In recent years, attention has focussed on more closely mimicking the *in vivo* environment. Prunieras, Regnier and Woodley (1983) recognised the potential importance of maintaining the cultures at the air-liquid interface such that the upper surface of the culture is exposed to the

air. This system is more akin to the *in vivo* situation than with conventional, submerged cultures and cells grown by this method possess many of the morphological characteristics of normal skin, including those which are absent in submerged cultures (Vaughan, Gray and Bernstein 1986).

An alternative approach was described by Hennings, Michael, Cheng, Steinert *et al.* (1980). In this system, the epidermal cells are maintained at a calcium concentration which is much lower than in conventional cultures. Low calcium cultures display uniformity and are very stable. They proliferate rapidly and form monolayers but do not stratify (*Ibid.*). This type of culture, by virtue of its homogeneity and stability, may be applicable to studies of, for example, drug action (Gilchrest 1986), as well as other factors which might affect growth or viability of epidermal cells.

Most of the culture systems described here were assessed to determine which one was appropriate for the envisaged study on factors affecting rat epidermal cell growth.

MATERIALS AND METHODS

PREPARATION OF EPIDERMAL CELL SUSPENSIONS

Epidermal cells were isolated from neonatal rat skin following either trypsin or dispase treatment. Donor rats were usually under 24 hours-old and always under 48 hours-old. Each time about ten rats were killed by lowering the body temperature and the bodies rinsed in 70 per cent ethanol and then in sterile PBS. The skin covering the trunk was excised, rinsed in sterile PBS and any adherent fat and connective tissue scraped off the underside with a scalpel; it was then cut into narrow strips of about 2x10 mm. Alternatively, the whole skin was stored in incomplete minimal essential medium (MEM) medium containing antibiotics (Appendix 4) overnight at 4°C and the procedure resumed the following day.

Preparation of Epidermal Cell Suspensions Using Trypsin

The strips of skin were submerged in a 0.25 per cent solution of trypsin in incomplete MEM (Appendix 4) and incubated for one hour at 37°C, or overnight at 4°C, both under humidified conditions. The trypsin was removed and replaced with MEM containing 20 per cent FCS to prevent further enzyme digestion. The epidermis was then peeled away from the dermis using forceps with the aid of a binocular microscope and shaken in medium for one minute to detach the basal cells. The remaining part of the epidermal strips was removed from the suspension using a sterile strainer and discarded. The cell suspension was centrifuged at 375g for ten minutes at room temperature and the cells resuspended in culture medium. The cell concentration and viability were determined by incubation of an aliquot with 0.06 per cent trypan blue in PBS for five

minutes at room temperature, followed by a count of dye-excluding and dye-incorporating cells with an improved Neubauer haemocytometer.

Preparation of Epidermal Cell Suspensions Using Dispase

The strips of skin were submerged in a 0.2 per cent solution of dispase (Appendix 4) and incubated at 4°C for 80-90 minutes. The epidermis was peeled away from the dermis and the epidermal strips incubated in a 0.25 per cent solution of trypsin in EDTA (Appendix 4) for 15 minutes at 37°C, with occassional agitation. The action of the trypsin was terminated by the addition of FCS and the strips gently agitated for ten minutes to allow further release of basal cells. The strips were then rinsed with PBS and the cell suspension collected and centrifuged at 400g for ten minutes at room temperature. Finally, the cells were resuspended in medium and the cell concentration and viability determined as above.

Density Gradient Separation of Epidermal Cells

A comparison was made between epidermal cultures seeded with cell suspensions prepared by the dispase method only and those seeded with basal cells which had been isolated from a Percoll discontinuous gradient following dispase cleavage.

Cells released from the epidermal strips after trypsin treatment, were spun down and resuspended in 1 ml PBS. This suspension, derived from a maximum of six rat skins, was layered on the top of a discontinuous Percoll gradient, with fractions of densities 0.09, 0.06, 0.03 and 0.01 g ml^{-1}, bottom to top (Appendix 4). A 1 ml suspension of density marker beads (Sigma) was layered on top of an identical gradient to allow the densities of separated cell bands to be deduced. The gradients were centrifuged at 1,250g for 50 minutes at 15°C. Each band of cells was

carefully harvested and washed twice in PBS (centrifuged at 400g, ten minutes, 15°C). The cells were resuspended in complete calcium-free MEM (SMEM) medium containing five per cent Myoclone FCS and 0.4 $\mu g\ ml^{-1}$ hydrocortisone (Appendix 4). Aliquots were taken for cytospins, whilst the remainder was used to seed 25 cm^{-2} tissue culture flasks (Nunclon). The cells were incubated at 37°C under a 95 per cent air-5 per cent carbon dioxide atmosphere and growth assessed daily. In addition, the rate of ^{3}H-thymidine incorporation of the separated basal cells was compared with that of epidermal cells, from the same rats, which had not been Percoll separated, when both were cultured under identical conditions in 96-well plates. The method used was that described on page 130.

INITIAL EPIDERMAL CELL CULTURE METHODS

Epidermal cell suspensions prepared by either the trypsin or the dispase method, either unseparated or as basal cell fractions from the Percoll gradient, were cultured under various conditions and in various culture media. These included incubation at 33°C or 37°C and culture on normal tissue culture plastic (Nunclon), on a culture surface reported to inhibit fibroblast growth (Primaria) or on a surface coated with collagen, prepared from rat skin by the method of Liu and Karasek (1978). Several culture media were tested for their suitability for rat epidermal cell growth. These were M199 (Appendix 4), MCDB-151 (Sigma), MEM or MEM containing D-valine rather than L-valine (MEM D-VAL, Gibco). The media were supplemented with 13-26 per cent FCS; other supplements tested were hydrocortisone and bovine brain extract, prepared from calf brain by the method of Maciag, Cerundolo, Ilsley, Kelley and Forand (1979). Epidermal cells were seeded at an initial cell density of

$1-5x10^5$ ml^{-1} and incubated under a 95 per cent air-5 per cent carbon dioxide atmosphere. The culture medium was removed and replaced with fresh medium at 24 hours and thereafter every two days.

EPIDERMAL CELL CULTURE ON SYNTHETIC MEMBRANES

As an alternative to the conventional submerged culture system, epidermal cells were fed with media from below, with the upper surface exposed to the air.

Epidermal cell suspensions were prepared by trypsin separation or by dispase cleavage followed by Percoll-gradient separation of the basal cells. The cells were seeded onto Millicell-HA membranes (Millipore) or Transwell membranes (Northumbria Biologicals), both with pore size 0.45 μm, at an initial cell density of $2x10^5$ cm^{-2}. The membranes were supplied as inserts for six well culture plates, such that two distinct compartments were created on either side of the membrane. M199 medium was supplemented with ten percent Myoclone FCS and 0.4 μg ml^{-1} hydrocortisone. Additionally, epidermal growth factor (EGF), insulin, cholera toxin and transferrin were tested for their effect on growth (Appendix 4). The cultures were incubated at 37°C, under a 95 percent air-5 percent carbon dioxide, humidified atmosphere. For the first seven days, medium was present in both compartments such that the cells were kept submerged. Thereafter, medium was only added to the external compartment such that the cells were fed from below. The culture medium was changed 24 hours after initiation and thereafter every two days. At intervals, cultures were sacrificed and submitted for sectioning by S.K. Onwuka and L. Inglis at the Moredun Research Institute, Edinburgh. The membranes, with attached cells, were fixed and embedded in epoxy

propane and epoxy resin. Sections, 1 μm thick, were stained with toluidine blue (Gurr).

EPIDERMAL CELL CULTURE IN LOW CALCIUM-CONCENTRATION MEDIUM

Epidermal cells were cultured in complete medium containing 0.2 mM calcium which was prepared from calcium-free MEM (SMEM). Initially, the medium was supplemented with 13 per cent Myoclone FCS. Myoclone contains approximately 4.6 mM calcium (product information), so prior to use the calcium was removed by treatment with Amberlite resin (BDH), following the chelex-treatment method of Swierenga and MacManus (1982). The complete medium was then supplemented with sterile calcium chloride (BDH) to give a final calcium concentration of 0.15-0.2 mM; this level was checked by submitting a sample for mass spectrophotometry. However, the cells grew equally as well in S-MEM supplemented with five per cent Myoclone as with 13 per cent, and this was the concentration routinely used. The final calcium concentration in the culture medium, containing five per cent Myoclone, was 0.2 mM; thus, there was no requirement for chelex treatment.

Epidermal cells were cultured in complete S-MEM (Appendix 4) in 25 cm^{-2} Primaria flasks, at an initial cell density of $2x10^{5}$ cm^{-2}, at 37°C under a 95 per cent air-5 per cent carbon dioxide atmosphere. The culture medium was changed at 24 hours and thereafter every two days.

Assessment of Medium Supplement Requirements of Epidermal Cells Cultured at Low Calcium Concentration

Skin from neonatal rats, under 24 hours-old, was split with dispase and the epidermis dissociated with trysin. The resultant cell suspension was separated into its component cell types on a Percoll discontinuous

gradient. The basal cell fraction was used to set up cultures in 96-well plates, at an initial cell density of $5x10^4 cm^{-2}$. The culture medium consisted of S-MEM with 0.75 g l^{-1} sodium bicarbonate, 20 mM Hepes buffer, 2 mM L-glutamine, 50 units ml^{-1} penicillin and 50 units ml^{-1} streptomycin sulphate. To this base, various combinations of the following supplements were added, 5 per cent FCS (Myoclone), 0.4 µg ml^{-1} hydrocortisone, 10 ng ml^{-1} EGF, 5 µg ml^{-1} insulin, 10 ng ml^{-1} cholera toxin and 5 µg ml^{-1} transferrin (Appendix 4). All supplements were of tissue culture grade.

Ten replicate cultures were set up for each treatment, avoiding the outside rows of the culture plates which were filled with sterile PBS. The cultures were incubated for 24 hours at 37°C under a 95 per cent air-5 per cent carbon dioxide, humidified atmosphere. The culture medium was then removed from each well and replaced with fresh medium of the appropriate type. After a further three days the medium was changed again and 0.5 µCi ^{3}H-thymidine in 50 µl complete medium, was added to each well. On day five of culture, 17 hours after addition of ^{3}H-thymidine, the cells were harvested. To harvest the cells, the medium was removed completely, since any remaining FCS might inhibit trypsin activity. Each well then received 0.2 ml of a 0.25 per cent solution of trypsin made up in PBS containing 0.02 per cent EDTA. The cultures were trypsinised for 30 minutes at 37°C, ignoring the possiblity of cell damage which was irrelevant since the cells would be lysed as a result of harvesting and the DNA collected on the filter. The cells were washed onto glass fibre paper using an automated cell harvester. Each filter paper disc, corresponding to the contents of one culture well, was placed in a scintillation vial with 1 ml scintillation fluid (Optiscint Hi-safe). The amount of incorporated ^{3}H-thymidine was determined with a liquid

scintillation counter and the median counts per minute calculated for each treatment.

COMPARISON OF EPIDERMAL CELL GROWTH IN LOW AND NORMAL CALCIUM CONCENTRATION MEDIA

Epidermal cells were cultured either with 0.2 mM or with 1.4 mM calcium in SMEM complete medium (Appendix 4) at an initial cell density of $2x10^5 cm^{-2}$. The culture method and determination of ^3H-thymidine incorporation was the same as for assessment of supplement requirements, except that both subconfluent and confluent cultures were assayed.

RESULTS

PREPARATION OF EPIDERMAL CELL SUSPENSIONS

Both 0.25 per cent trypsin and 0.2 per cent dispase proved effective in detaching the epidermis from the dermis. Dispase separation yielded a significantly higher percentage of viable cells than did trypsin (Mann-Whitney U^{16}_{16}=16, $P < 0.01$, table 5.1). In addition, the yield of viable epidermal cells per rat was significantly greater with dispase than with trypsin separation (Mann-Whitney U^{16}_{16}=16, $P < 0.01$, table 5.2).

It became apparent that some fibroblasts were carried over into the epidermal cultures when the rat skin was separated with trypsin, whereas fibroblasts were a rare contaminant of the epidermal cultures derived from dispase separated skin. When fibroblasts became incorporated into the epidermal suspensions used to seed cultures, they proliferated more quickly than the epidermal cells and became the dominant cell type within about ten days.

The epidermal cell suspensions harvested from non-Percoll purified dispase-separated skin, consisted of about 80 per cent small or medium, round, viable cells, 10 per cent large, differentiated viable cells and 10 per cent non-viable cells, both large and small (Figure 5.1). The small round cells had the characteristic appearance of basal cells, the proliferative cells of the epidermis. The larger cells were of irregular shape, contained granules and were characteristic of the non-proliferating epidermal cells situated above the basal layer. The non-viable cells were a mixture of small and large cells, some of which would have been killed during the separation procedure and the rest which would have been *stratum corneum* cells, which are non-viable *in vivo*.

Table 5.1: Percentage viable epidermal cells obtained from trypsin and dispase-separated skin

Method	Viability (%)	Range	n
Trypsin, 37°C	64	42-87	31
Trypsin, 4°C	77	59-81	7
Dispase	91	81-96	16
Dispase + gradient separation[1]	95	71-98	5

Values are medians of n determinations.
[1]Figures apply to the cells harvested from the most dense fraction of the gradient

Table 5.2: Yield of viable epidermal cells obtained from trypsin and dispase-separated skin

Method	Cells per rat ($\times 10^6$)	Range	n
Trypsin, 4°C	7	1-7	7
Dispase	10	5-14	17
Dispase + gradient separation[1]	8	5-11	5

Values are medians of n determinations.
[1]Figures apply to the cells harvested from the most dense fraction of the gradient

Separation of the dispase-treated epidermal cells on a Percoll density gradient resulted in four fractions, each containing a predominant cell type.

The most dense layer of the Percoll gradient (0.09 g ml^{-1}) contained about 92 per cent small or medium sized, non-granular cells, 3 per cent large, granular cells and 5 per cent non-viable cells (Figure 5.2). When culture flasks were seeded with this fraction, confluent cultures formed within six days.

The second most dense layer of the Percoll gradient (0.06 g ml^{-1}) contained about 84 per cent small or medium sized cells and 16 per cent large, granular cells. Cultures also formed from this fraction, although they did not become confluent.

The 0.03 g ml^{-1} fraction contained about 66 per cent large, granular cells and 34 per cent medium non-granular cells. Although a few of these cells attached to the culture flask, they detached after a few days and did not proliferate.

The 0.01 g ml^{-1} fraction contained very few cells together with debris; the cells were small and round and attached to the culture flask but remained rounded up and did not spread out or proliferate.

Cell cultures, using Percoll-separated cells, were initiated with the 0.09 g ml^{-1} fraction. According to the median values, derived from a large number of determinations, cells from this fraction did not possess a significantly higher viability than the unfractionated suspension; neither, apparently, did the separation result in significantly fewer cells (Tables 5.1 and 5.2, Mann-Whitney, $P > 0.05$). However, when the number of small viable cells harvested from a gradient were compared with the number of small, viable cells layered onto it, it was clear that cells were lost during the density separation and the overall yield was

about 54 per cent.

Epidermal cell cultures derived from dispase-separated skin and either Percoll purified or not, were assayed for ^{3}H-thymidine incorporation after being cultured under identical conditions and with the same initial cell density (p.130). The median CPM of non-Percoll purified cultures was 10,000 (range 6,000 to 11,000), whilst the median CPM of Percoll purified cultures was 4.000 (range 3,000 to 6,000). These values were the medians of ten replicates, rounded to the nearest 1,000. The ^{3}H-thymidine incorporation rate of the non-purified cultures was significantly greater than that of the cultures deriving from the Percoll gradient (Mann-Whitney U^{10}_{10}=16, $P < 0.01$)

INITIAL EPIDERMAL CELL CULTURE METHODS

Epidermal cells in complete medium, containing 13-26 per cent FCS and 0.4 $\mu g\ ml^{-1}$ hydrocortisone formed confluent cultures within three to six days. The cultures began to stratify, forming multilayered structures in areas of confluence (Figure 5.3). Whilst areas of stratification were forming in the culture, other areas were usually beginning to detach. Often, only the upper layers detached leaving a monolayer behind. On a few occasions, a confluent sheet of multilayered culture formed and remained for several days. For most cultures a steady state was never reached with different areas proliferating, stratifying or detaching at the same time. Thus, epidermal cell cultures most commonly appeared as uneven stratified cultures, with monolayers in between the areas of multilayering (Figure 5.4). The lifespan of the cultures varied from ten days to over a month, with a shift towards detachment towards the end of this time. In many cultures, particularly those seeded with cells prepared by trypsin separation, fibroblasts often became numerous and

eventually outgrew the epidermal cells.

Of the various culture conditions tested, the presence of hydrocortisone in the culture medium seemed to be the only essential additional ingredient for establishment of the cultures. The cells grew at 33 and 37°C, though more slowly at 33°C; the culture lifespan was similar for both. The Primaria flasks appeared to be less favourable to fibroblast growth than the normal Nunclon culture surface and so, were routinely used. Nevertheless, the Primaria flasks did not totally inhibit fibroblast growth. The medium MEM D-VAL also did not totally inhibit fibroblast growth. The epidermal cells grew equally well in M199 and MEM, whereas medium MCDB did not support growth well. Supplementation of the complete medium with bovine brain extract did not enhance epidermal cell growth. Finally, although the cells attached well to a collagen-coated flask, subsequent growth was not superior to that of uncoated Primaria flask cultures.

EPIDERMAL CELL CULTURE ON SYNTHETIC MEMBRANES

The epidermal cells attached well to both types of synthetic membrane. Sections of the Transwell cultures revealed that after seven days one to two cell layers had formed on the membrane. When the cultures were fed from below only with full supplement-containing medium, a *stratum corneum*-like structure developed in ten to fourteen days. These layers, which resembled keratin, overlaid two to three layers of cells, the upper ones of which were flattened (Figures 5.5 and 5.6). Up to seven layers of the keratin-like material was present on day 21, although by this stage the underlying cells appeared necrotic. In two out of three experiments, this type of culture formed. On the third, one to three layers of cells grew but the keratin-like layers were absent.

When the cultures remained submerged in medium and also where the medium lacked cholera toxin and transferrin, only two layers of cells were present, with the upper layer consisting of flattened cells. Under these conditions the keratin-like layers did not form (Figure 5.7).

The Transwell membranes proved superior to the Millicell membranes. In the latter, two to three layers formed, with the upper layers appearing necrotic. These cells often formed into large, degenerating clumps and keratin-like layers were never visible.

Epidermal growth was also assessed on Transwell membranes with pore size of 3.0 μm, as opposed to 0.45 μm. Although the cultures were stable for nineteen days before cell detachment became obvious, examination of the sections revealed that the cells were growing on both sides of the membrane. One to two cell layers were present on the upper surface and one layer was present on the underneath of the membrane; cellular processes were visible within the pores of the membrane, extending from one side to the other.

EPIDERMAL CELL CULTURE IN LOW CALCIUM-CONCENTRATION MEDIUM

Epidermal cells seeded in medium containing 0.2 mM calcium, proliferated to form confluent cultures within three to five days. The culture appearance was strikingly different from that of epidermal cells in normal calcium-concentration medium. At all times, growth was confined to a monolayer composed of small, regular cells with a characteristic basal cell appearance (Figure 5.8). In contrast to growth in normal calcium concentrations, the cultures in low calcium reached a steady state with a confluent monolayer maintained for at least two to three weeks. In addition, these stable, uniform cultures were highly reproducible.

The comparatively low concentration of calcium appeared to cause no permanent changes in the epidermal cells. If the calcium level of these cultures was raised to the normal concentration the cultures began to stratify and form uneven, multilayered structures typical of cultures initiated in this medium.

Assessment of Medium Supplement Requirements by Epidermal Cells Cultured at Low Calcium Concentration

Two medium supplements were found to be essential for epidermal cell growth under the culture conditions described; these were FCS and hydrocortisone. In the presence of these together, large areas of confluent monolayer were visible in the day five cultures, which incorporated significant amounts of ^{3}H-thymidine. In contrast, in the absence of FCS and hydrocortisone, no growth of the cells occurred and this was reflected by a lack of ^{3}H-thymidine uptake. Likewise, when the medium was supplemented with either FCS or hydrocortisone alone, no growth was visible, nor were significant amounts of ^{3}H-thymidine incorporated, the CPM values being similar to background. Determination of statistical significance was by means of the Mann-Whitney test ($U^{1}_{8}=16$, $P < 0.01$).

In the presence of FCS, further supplements were assayed for stimulation of epidermal growth; these were EGF, insulin, cholera toxin and transferrin. None of the other supplements could substitute for the stimulatory effect of hydrocortisone, giving CPM values at the background level (Table 5.3). However, when cholera toxin was added together with either insulin or transferrin, a small, but significant (Mann-Whitney $U^{1}_{8}=16$, $P < 0.01$) increase in thymidine uptake occurred (Table 5.3).

In the presence of hydrocortisone and FCS together, addition of either

EGF or transferrin made no significant difference to ^{3}H-thymidine uptake. In contrast, the addition of cholera toxin or insulin significantly increased the amount of ^{3}H-thymidine incorporated (Mann-Whitney $U^{1,8}_{1,0}=16$, $P < 0.01$), with the addition of cholera toxin causing almost a ten-fold increase in uptake (Table 5.3). Epidermal cells in medium containing FCS, hydrocortisone and cholera toxin grew well and incorporated ^{3}H-thymidine at a relatively high rate. Further addition of a combination of insulin, transferrin and EGF did not lead to an increase in the rate of ^{3}H-thymidine uptake (Table 5.3); neither did it lead to a significant decrease (Mann-Whitney $U^{1,8}_{1,0}=23$, $P > 0.05$).

COMPARISON OF EPIDERMAL CELL GROWTH IN LOW AND NORMAL CALCIUM-CONCENTRATION MEDIA

In the first experiment, epidermal cultures grown in either low or normal calcium medium, were assayed for ^{3}H-thymidine incorporation, on day four when both types of culture were confluent. The cells cultured in low calcium medium incorporated ^{3}H-thymidine at three times the rate of, otherwise identical, cells in normal calcium medium (Table 5.4, Mann-Whitney $U^{1,8}_{1,0}=16$, $P < 0.01$).

In the second experiment, both types of culture were still subconfluent when assayed for ^{3}H-thymidine incorporation on day four of culture. In contrast to the results for confluent cultures, there was no significant difference between uptake in low and normal calcium cultures (Table 5.4, Mann-Whitney $U^{1,8}_{1,0}=16$, $P < 0.01$).

In a third experiment, enough replicates, from the same batch of epidermal cells, were set up such that ^{3}H-thymidine uptake could be determined both before and after the cultures had attained confluence. The results bore out those from the previous two experiments. A

comparison of growth in low or normal calcium medium showed there was no difference in ^{3}H-thymidine uptake by subconfluent cultures, whereas a significant difference occurred with the confluent cultures (Mann-Whitney $U^{8}_{8}=16$, $P < 0.01$). The cells in low calcium medium incorporated almost three times as much ^{3}H-thymidine as those in normal calcium medium (Table 5.4).

Table 5.3: ^{3}H-thymidine incorporation by epidermal cells in the presence of various medium supplements

Supplement	Median CPM x 10^{2}	Range
None	0	0
Hydrocortisone	5	1-15
EGF	0	0
Insulin	0	0-1
Cholera toxin	0	1-2
Transferrin	0	0
EGF, insulin	0	0
EGF, cholera toxin	0	0
EGF, transferrin	0	0
Insulin, transferrin	0	0-1
Insulin, cholera toxin	1	0-1
Cholera toxin, transferrin	1	0-2
Hydrocortisone, EGF	3	1-11
Hydrocortisone, insulin	9	4-23
Hydrocortisone, cholera toxin	43	4-79
Hydrocortisone, transferrin	3	1-5
Hydrocortisone, EGF, insulin, cholera toxin, transferrin	38	12-68

The medium contained 5 % FCS. Values are the medians of ten replicates, rounded to the nearest 100. The median background radiation was 12 CPM, this was subtracted from the samples. All the tabulated CPM values, above zero, were significantly greater than that for FCS alone (Mann-Whitney $U^{10}_{10}=16$, $P < 0.01$). The supplements were included at the concentrations detailed on page 130

Table 5.4: ^{3}H-thymidine incorporation by epidermal cells cultured in low or normal calcium medium

Expt.	Time (h)	State of culture	Median CPM x 10^{3} 0.2 mM calcium		1.4 mM calcium		statistical significance
1	96	confluent	25	(19-33)	8	(7-11)	P<0.01
2	96	subconfluent	9	(3-11)	8	(6-13)	NS
3	24	subconfluent	1	(0)	1	(0)	NS
3	48	subconfluent	8	(6-9)	9	(8-10)	NS
3	72	confluent	16	(13-17)	6	(5-7)	P<0.01

Values are rounded to the nearest 1,000 and are medians of 16, 12 and 10 replicates (experiments one, two and three respectively) with the range given in brackets. The significance was determined with the Mann-Whitney test. NS represents not significant.

DISCUSSION

An essential requirement for the initiation of epidermal cell cultures is a source of cells which have high viability and are free from contamination by other cell types. Several methods for the isolation of epidermal cells for cell culture were tested. Both trypsin and dispase were effective in separating the epidermis from the dermis. Trypsin separation has been the most frequently used technique, in this respect (Skerrow and Skerrow 1985). At 4°C trypsin cleaves the skin at the dermal/epidermal junction, whereas at 37°C, basal and spinous cells dissociate from the epidermal sheet as well as the epidermal/dermal cleavage (*Ibid.*).

In recent years dispase, a neutral protease produced by *Bacillus polymyxa*, has been has been introduced for use in cell culture (Kitano and Okada 1983). This enzyme separates the skin at the dermal/epidermal junction; the epidermis can then be dissociated by short-term trypsin treatment at 37°C. Dispase has a very low toxicity, with 95 per cent viability of epidermal cells isolated by this method (*Ibid.*). The present study lends support to this work, since epidermal cells isolated from skin by dispase treatment had a significantly higher level of viability than cells isolated from trypsin-separated skin. The results also support the claim by Kitano and Okada (*Ibid.*) that fibroblast contamination of the epidermal cell suspension is minimal. In addition, significantly higher yields were obtained with dispase than with trypsin-separated skin.

Epidermal suspensions obtained from dispase-separated skin consisted of about 80 per cent cells with a characteristic basal cell appearence. In an attempt to increase the purity of the basal ie. proliferative cells,

the suspension was centrifuged on a Percoll discontinuous gradient. Different cell types preferentially localised in each of the four Percoll fractions. The majority of cells resembling basal cells were found in the most dense fraction of 0.09 g ml^{-1}, in agreement with the work on separation of newborn rat epidermal cells by Brysk, Snider and Smith (1981). The density gradient separation inevitably led to a decrease in the overall yield of basal cells. Furthermore, the Percoll-purified basal cells appeared not to grow in culture so well as the non-purified cells, as evidenced by the significantly lower ^{3}H-thymidine incorporation rate. Morhenn, Starr, Terrell, Cox and Engleman (1982) separated human epidermal cells on a density gradient and found that the basal cell fraction had the highest growth rate of the various fractions but nevertheless, did not grow as well as unseparated epidermal cells. The authors concluded that other epidermal cell types were necessary for optimal basal cell growth.

Most of the differentiated epidermal cells did not attach and grow in culture (p.133) and so were removed with the first change of media. Vaughan and Bernstein (1971) found that only the basal cells grew in culture, whilst other epidermal cells initially attached to the culture flask, but did not proliferate and were removed on changing the culture medium. Thus, the remaining justification for including the density gradient separation would be to decrease fibroblast contamination. With dispase-separated skin very few fibroblasts are carried over into the epidermal cell suspension. Fibroblasts were not observed in short-term epidermal cultures, so the gradient separation step was omitted for these (Chapter 6). However, for longer-term cultures, this purification step was included (p.129).

Additional methods for preventing fibroblast growth proved unecessary

and inefficient. Culture medium containing D-valine, in the place of L-valine, did not inhibit fibroblast growth, in contrast to the claim by Gilbert and Migeon (1975), who reported that epithelial cells but not fibroblasts could convert D-valine to the essential L-valine. It is possible that the medium was not inhibitory due to the presence of L-valine in the medium supplement, FCS.

The most commonly used incubation temperature for epidermal cell culture has been 37°C. Some authors have claimed that a longer culture lifespan results at 33°C for mouse cells (Marcelo, Kim, Kaine and Vorhees 1978) and 30-32°C for rat epidermal cells (Indo and Wilson 1977). In the present study, the culture lifespan for neonatal rat epidermal cells was similar at 33°C and 37°C. Likewise, although human epidermal cells have been reported to attach to a collagen-coated surface with five times greater efficiency than to a normal culture surface (Liu and Karasek 1978b) little subsequent effect on the growth and differentiation of rat epidermal cells was observed. The one additional factor which enhanced the establishment and growth of the cultures was hydrocortisone. This has been widely used as a medium supplement for epidermal cell cultures to promote growth (Rheinwald and Green 1975) and differentiation (Tammi and Santti 1982).

Limited success was achieved with this conventional type of culture system. Both proliferation and differentiation into multilayered structures occurred, but, in general, the cultures lacked uniformity and stability. Similar results were reported by Fusenig and Worst (1975) for neonatal mouse epidermal cell cultures where stratification did not extend over the entire culture surface and where the culture lifespan was about two weeks after which detachment occurred.

A second approach involved a culture system more akin to the *in vivo*

situation. Here, the cultures were seeded on synthetic membranes and fed from below, with the upper surface exposed to the 95 percent air-5 percent carbon dioxide atmosphere. Vaughan *et al* (1986) described the growth of rat epidermal cells on a specially constructed nylon membrane. After seven days of submerged culture, the membrane was raised to the air-liquid interface. By day 12, the cultures consisted of approximately three cell layers, on top of which one to two enucleated layers, reminiscent of the *stratum corneum*, had formed. By day 15, 15-25 cornified layers were present. In contrast, cultures which were kept submerged did not develop the cornified layers. Although Vaughan *et al* (1986) found the other types of membrane they tested gave inferior results, the Transwell membranes used in this study, proved quite successful in this respect. Terminal differentiation of the cultures took place, with the development of up to seven cornified layers. In agreement with the findings of Vaughan *et al* (1986), these layers did not form when the cultures remained submerged.

It became clear that further development of this culture system was required to overcome instability problems. For reasons of limitation of time, this was not pursued. However, the air-liquid method of culture does possess great potential, both for studies of the effect of factors released from stimulated mononuclear cells on epidermal differentiation and as a model for infection of the skin by *D.congolensis*. Here, the course of infection could be studied, following application of *D.congolensis* to the surface of the culture, under a variety of experimental regimes.

A further development would be culturing epidermal cells on a "dermal equivalent" composed of a collagen gel containing living fibroblasts. Of all the epidermal culture systems, this one most closely mimics *in situ*

skin. All the differentiation markers present in normal skin can be identified in the cultured epidermis, although several differences in their position and orientation occur (Asselineau, Bernard, Bailly, Darmon and Prunieras 1986). A disadvantage with this type of culture is that epidermal cells are not isolated from other cell types; thus any response of the epidermal cells to added factors may be an indirect one mediated via the fibroblasts.

Producing a differentiating epidermal cell culture, with the same characteristics as *in vivo*, has proved a difficult task. If differentiation could be dissociated from proliferation, an alternative approach could be made, whereby the effects of factors from stimulated mononuclear cells on epidermal proliferation rates could be studied. This can be achieved using low calcium-concentration medium. Maintenance of rat epidermal cells in medium containing 0.2 mM calcium produced monolayer cultures, with a high degree of uniformity and stability. These results are in agreement with the findings of Hennings *et al* (1980) for mouse cells except for a slight difference in the calcium concentration which prevented stratification. This was 0.05-0.1 mM for the mouse cells, with 0.2 mM calcium causing small areas of stratification. The exact mechanism by which differentiation is controlled by calcium is unknown; some products of differentiation are found in low calcium cultures such as keratin proteins. However the full programme of differentiation only occurs when the cultures are transferred to normal calcium concentration medium (*Ibid.*)

The proliferation rates of rat epidermal cells in low and in normal calcium medium were assessed, by means of a ^{3}H-thymidine incorporation test. Confluent cultures in low calcium medium had much higher incorporation rates than those in normal calcium medium, in agreement

with the results for mouse cells, reported by Hennings *et al* (1980). Thus, reduction in the extracellular calcium concentration, resulted in continuation of high proliferation rates and the prevention of differentiation. In contrast, subconfluent rat epidermal cells incorporated ^{3}H-thymidine at similar rates in low or normal calcium medium. This result is not surprising since, in a subconfluent culture, under favourable conditions, the cells will be in the exponential growth phase, regardless of the calcium concentration. It is only when confluence is attained that the shift from proliferation to differentiation occurs in normal calcium cultures (Holbrook and Hennings 1983) whereas, the shift never occurs in low calcium cultures (Hennings *et al* 1980).

The same assay was used to determine which medium supplements would enhance epidermal growth and should therefore be included in the culture medium. All supplements were tested at the concentration which has been most commonly used by other investigators. Two components were found to be essential to the growth of the cultures, namely FCS and hydrocortisone. FCS is commonly included in epidermal cell culture medium at 5-20 percent, although a few reports have claimed good growth in serum-free medium. Boyce and Ham (1985) described growth of human epidermal cells in serum-free, low calcium medium, with patchy stratification in normal calcium medium.

Hydrocortisone has been widely used in epidermal cell culture, usually at a concentration of 0.4 $\mu g\ ml^{-1}$, which was the concentration used here. The proliferation rate of rat epidermal cells was increased in the presence of hydrocortisone, in agreement with the report by Vaughan, Kass and Uzman (1981). The mechanism by which hydrocortisone increases proliferation of cultured cells is unknown; one suggestion is that it

aids in the repair of cell damage, this could be important in the initial setting up of cultures after an unfavourable isolation procedure (Vaughan, Kass and Uzman 1981).

Of the additional supplements tested, the inclusion of cholera toxin and of insulin resulted in higher proliferation rates of the epidermal cells. Cholera toxin stimulates the production of cyclic adenosine monophosphate (AMP) by its action on the enzyme adenyl cyclase (Stryer 1981). Elevation of cyclic AMP has been shown to stimulate increased cell proliferation and differentiation of cultured, neonatal mouse epidermal cells (Marcelo and Tomich 1983). Cholera toxin was included at a concentration of 10 ng ml^{-1}, as used by, among others, Heimann and Rice (1983). Insulin has been reported to enhance rat epidermal cell proliferation, but only in the presence of hydrocortisone (Vaughan *et al* 1981). The exact mode of action is not certain, but it is believed insulin enhances protein synthesis (Schwartz and Amos 1968).

Transferrin appeared to have no stimulatory effect on its own but it had a very slight one in the presence of cholera toxin. Although transferrin contributed little to the cultures, it was an important ingredient of the medium for the differentiating cultures on synthetic membranes described earlier. EGF did not stimulate epidermal proliferation at the concentration tested, which is that most widely used. The information regarding the effect of EGF on epidermal cells is conflicting. Epidermal cells possess a membrane receptor for EGF (O'Keefe, Battin and Payne 1982) and some stimulation of epidermal growth, in the absence of other cell types, has been reported (Liu, Eaton and Karasek 1979). However, Peehl and Ham (1980) found, as here, that EGF did not promote epidermal growth in the absence of a feeder layer.

In conclusion, maintenance of epidermal cells in low calcium-

concentration medium results in the most stable and reproducible cultures. However, short-term cultures of epidermal cells in normal calcium concentration medium showed the same proliferative capabilities as those in low calcium medium, provided that the cultures remained sub-confluent. A low calcium culture system was initially chosen for an assessment of the effect on epidermal cell proliferation of factors released from stimulated mononuclear cells.

FIGURE 5.1 Dispase-separated epidermal cells, pre-purification.

Giemsa-stained cytospin showing:
Small, undifferentiated cells (closed arrow)
Large, differentiated, granular cells (open arrow)

(x 425 magnification)

FIGURE 5.2 The most dense fraction taken from a Percoll gradient on which the dispase-separated cells (Figure 5.1) were layered.

Giemsa-stained cytospin showing:
Small, undifferentiated cells (closed arrow)
Medium, undifferentiated cells (open arrow)

(x 425 magnification)

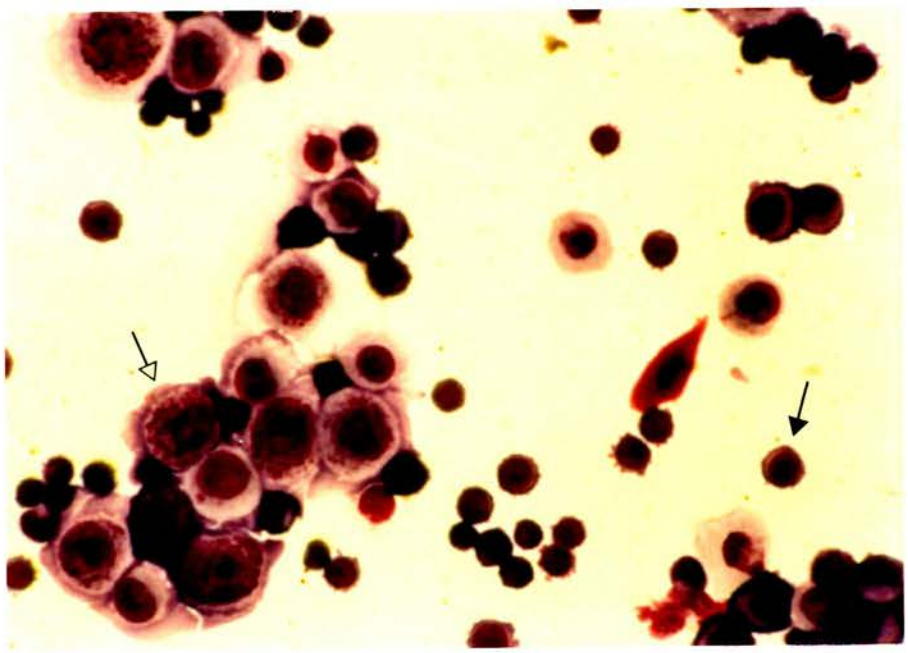
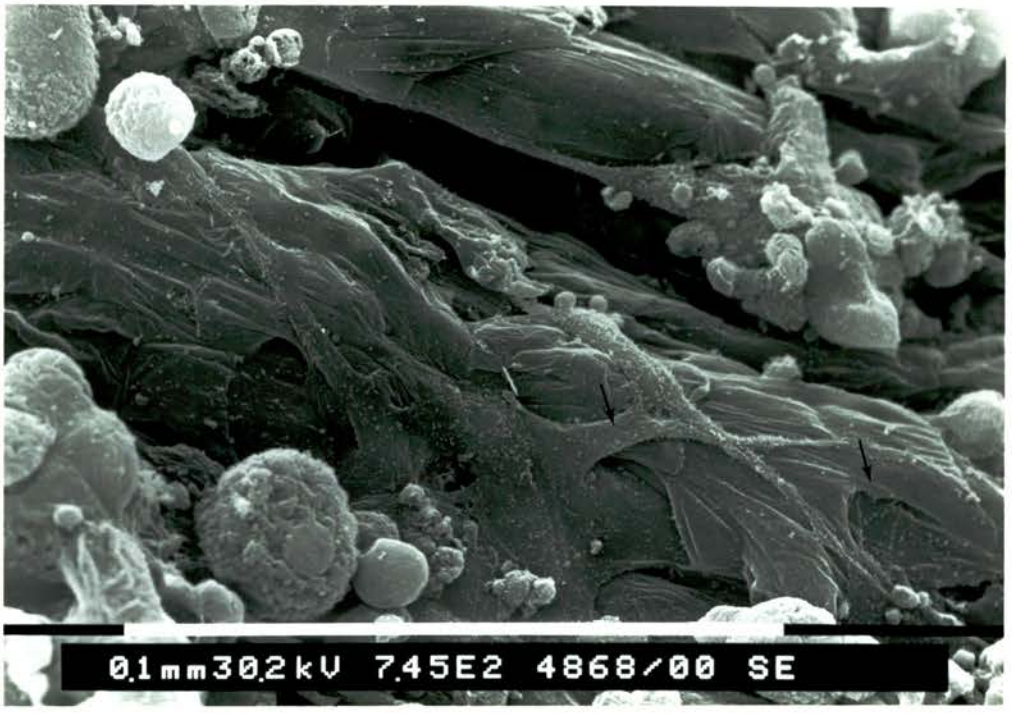
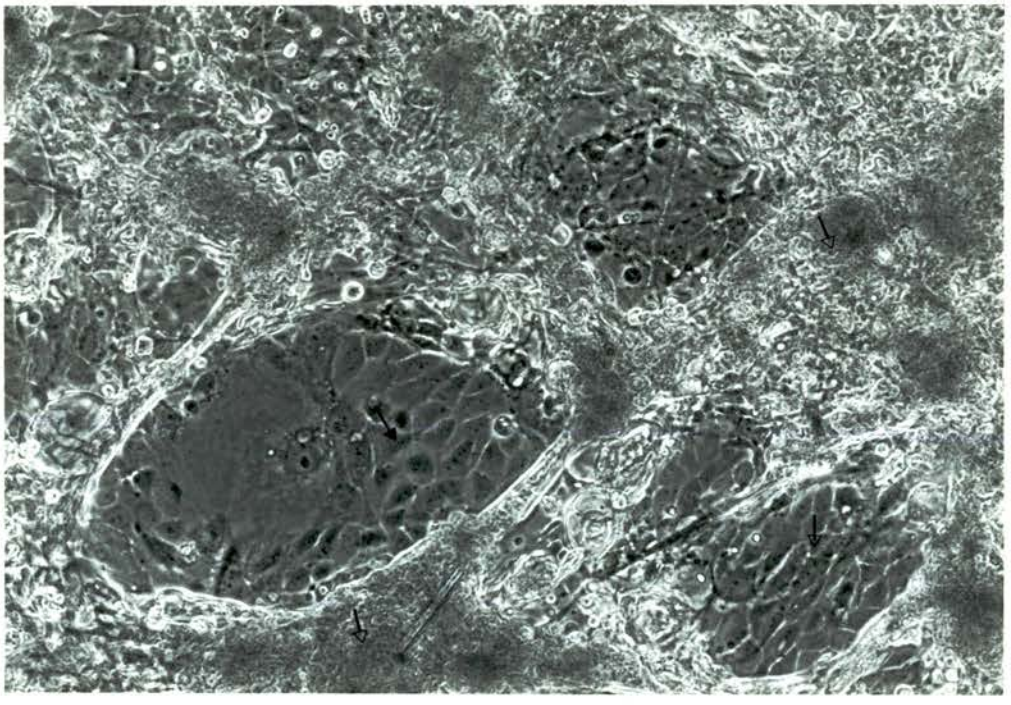


FIGURE 5.3 Scanning electron-micrograph of a stratified epidermal cell culture on day seven. Multi-layering is indicated by the overlying cells (arrows). Large spheres are rounded-up epidermal cells.

FIGURE 5.4 Epidermal culture after ten days in normal calcium-concentration medium.

Phase contrast showing:
Areas of stratification (open arrows)
Areas of monolayer (closed arrows)
(x 210 magnification)


0.1mm30.2kV 7.45E2 4868/00 SE



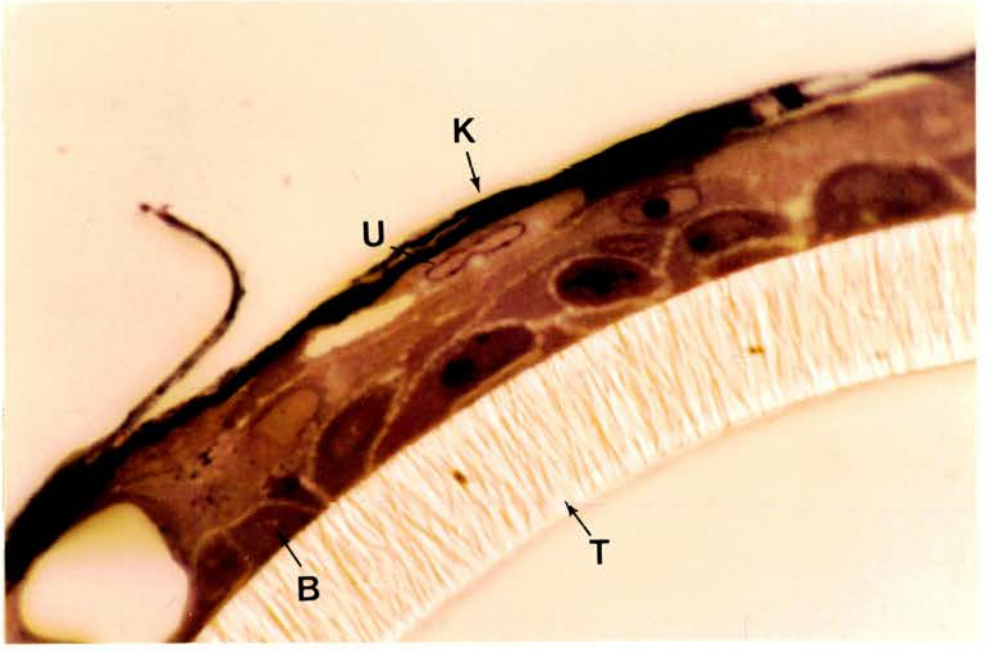
FIGURES 5.5 and 5.6 Epidermal cultures grown at the air-liquid interface on Transwell membranes for fourteen days.

Toluidine blue-stained sections showing:

K	Keratin-like material
U	Upper-layer, flattened cell
B	Basal-layer cell
T	Transwell membrane

Figure 5.5 x 425 magnification
Figure 5.6 x 1,060 magnification

5.5



5.6

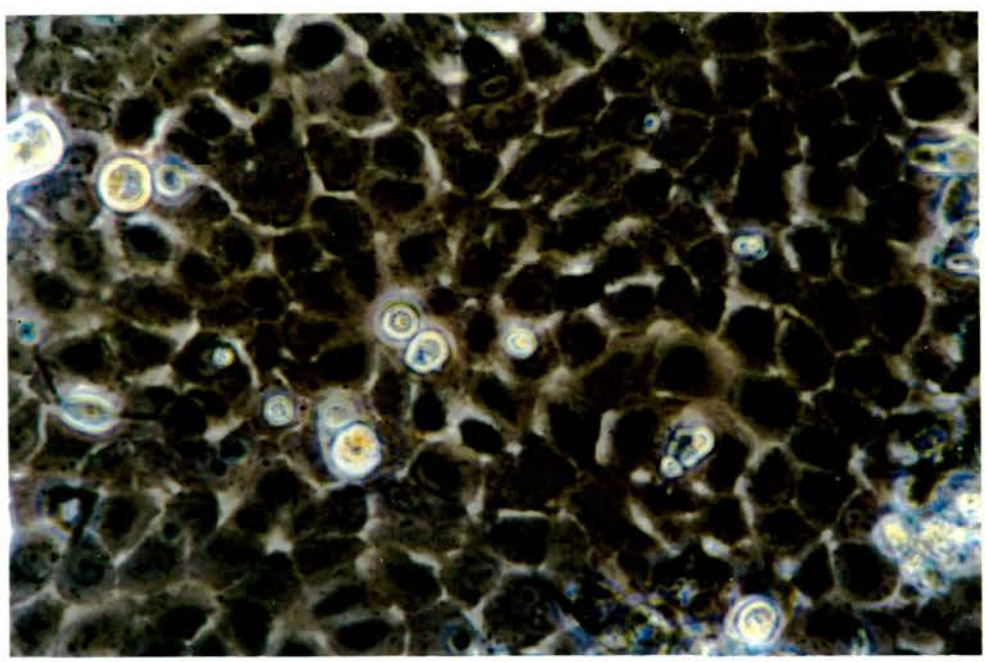
FIGURE 5.7 Epidermal culture grown submerged in medium on a Transwell membrane for fourteen days.

Toluidine blue-stained section showing two to three cell layers. Note the absence of the keratin-like layer.

(x 425 magnification)

FIGURE 5.8 Epidermal cells cultured under low calcium-concentration conditions.
Phase contrast showing confluent monolayer on day fourteen.

(x 340 magnification)



CHAPTER SIX

EFFECT OF LYMPHOKINE-CONTAINING SUPERNATANTS ON EPIDERMAL CELL PROLIFERATION

CONTENTS

INTRODUCTION

Dermatophilosis lesions in transient infections, such as those which are experimentally-induced are characterised by invasion and partial destruction of the epidermis, with the formation of a new epidermis underneath (Oduye 1976b). In natural, chronic infections the process is repeated with invasion of the new epidermis (Stewart 1972a).

The epidermis which forms after *D.congolensis* infection displays acanthosis and hyperkeratosis and is therefore much thicker than normal, possibly as a result of increased epidermal proliferation. Amakiri (1974) proposed that the changes were a means of eliminating the organism, a view supported by the earlier work of Zlotnik (1955) who suggested that increased epidermal proliferation at the site of infection was caused by the observed lymphocytic infiltrate. Thus, epidermal hyperproliferation may be caused by a combination of immune-mediated and non-specific mechanisms.

The potential role of the epidermis in active defence against invading pathogens has been recognised in the field of human dermatophyte infections. Rothman and Lorincz (1963) discussed previous observations that individuals whose skin had a lower than normal desquamation rate were more susceptible to chronic dermatophyte infections, noting that the desquamation rate may be an important component of host defence.

Berk, Penneys and Weinstein (1976) also proposed that epidermal turnover is an important defence mechanism. They showed that at the active edge of a ringworm infection, a higher than normal proportion of epidermal cells were in a state of cell division and this was associated with epidermal thickening. Tagami (1985) observed increased epidermal proliferation in response to *Trichophyton mentagrophytes*-infection and

found that the peak of proliferation correlated with the onset of resolution of the infection and with the occurrence of the inflammatory response. Primary infections were characterised by maximal epidermal proliferation and thickness on day 11, which was also the time when the lesion began to resolve. An accelerated response occurred on re-infection with maximal epidermal proliferation attained on day two; again this coincided with the onset of healing and of the inflammatory response.

Sohnle, Frank and Kirkpatrick (1976) demonstrated that guinea-pigs, experimentally infected with *Candida albicans*, underwent pronounced scaling, ie. desquamation, at the infection site, with concomittant shedding of the organism. Animals which had been previously exposed to *C.albicans* underwent a delayed-type hypersensitivity reaction on reinfection and this was associated with enhanced scaling and faster clearance of the organism from the skin than in naive animals. The authors postulated that the lymphocytes, visible in the upper dermis under the infection site, released a lymphokine which increased the turnover rate of the epidermis by causing either an increase in the epidermal proliferation rate or an increase in the rate of keratinisation. In a later report, Sohnle and Kirkpatrick (1978) extended their findings in demonstrating that the percentage of proliferating epidermal cells at a *C.albicans*-infection site was significantly greater in an immune than in a naive animal. In addition they showed that non-specific inflammatory reactions to, for example, intradermally-injected ethanol could also cause increased epidermal proliferation. Kragballe, Desjarlais and Voorhees (1985) found that leukotrienes were stimulatory for epidermal proliferation and proposed that large amounts of these factors would be released at inflammatory sites largely by PMN.

Investigation of the potential role of the epidermis in host defence is hindered by the functional complexity of the skin. The epidermis is a stratified structure consisting of both proliferating and differentiating compartments. The proliferating stem cells are confined to the basal layer where they comprise a small proportion of the total cells (Lavker and Sun 1983). The stem cells give rise to daughter cells which lie immediately above the basal layer and which have a limited capacity to divide (*Ibid*). As cells leave the basal layer they become committed to differentiation; this progresses as the cells migrate up through the *stratum spinosum* and *stratum granulosum* to the *stratum corneum*, from which the cornified cells are eventually shed (Plumb and Wright 1985).

In normal skin a steady state of cell proliferation and desquamation exists. It has been suggested that maintenance of the homeostatic state is due to the constitutive production of mitotic inhibitors, chalones, by the mature epidermal cells. In this model, epidermal damage would lead to a decrease in chalone production, with concomittant increased proliferation until the epidermis returns to its steady state (Marks, Bertsch and Schweizer 1978).

Three components define epidermal kinetics, the rate of cell production, the rate of transit through the layers and the rate of cell loss from the surface. In turn, the rate of cell production depends on both the proportion of cells dividing and the rate of proliferation (Plumb and Wright 1985). Although it is not possible to determine absolute values for all three parameters, valuable information can still be gained in comparative studies of, for example, normal and diseased skin (*Ibid*).

In this study the rate of epidermal cell proliferation was determined in the presence of culture supernatants derived from *D.congolensis*-

stimulated mononuclear cells and compared with the proliferation rate in the presence of control supernatants. A comparison was made between culture supernatants derived from infected rats and those from naive rats to determine whether any observed effects were caused by factors specific to *D.congolensis* infection. The first set of experiments utilised epidermal cells cultured in low calcium-concentration medium because of the superiority of these cultures over normal calcium concentration cultures (Chapter 5). However, due to the many physiological roles of calcium, the experiments were then repeated using normal calcium-concentration medium to eliminate any behavioural artefacts caused by low availability of calcium. The experiments were conducted over a period of 72 hours after initiation of culture and therefore, problems with the inherent instability of the normal calcium cultures were not encountered.

MATERIALS AND METHODS

EPIDERMAL CELL CULTURES

Epidermal cell suspensions were prepared from neonatal rat skin, by the dispase-separation method (p.127). The isolated cells were resuspended in S-MEM medium containing five per cent Myoclone FCS, 0.4 $\mu g\ ml^{-1}$ hydrocortisone, 10 $ng\ ml^{-1}$ cholera toxin, 0.75 $mg\ ml^{-1}$ sodium bicarbonate, 20 mM Hepes buffer, 2 mM L-glutamine, 50 $units\ ml^{-1}$ penicillin and 50 $\mu g\ ml^{-1}$ streptomycin sulphate (Complete S-MEM medium, appendix 5). An aliquot was taken for cell and viability counts (p.126) and the cell concentration adjusted to $3.8x10^{5}\ ml^{-1}$ with complete medium. The suspension was seeded into 96-well, flat-bottomed plates, during which time, gentle agitation ensured that the cells remained evenly distributed. Each well, area 0.38 cm^2, received 200 μl of suspension to give an initial cell density of $2x10^{5}\ cm^{-2}$. The appropriate number of cultures was set up, such that there would be ten replicates for each treatment. The outer wells of the plates were filled with sterile PBS and the cultures incubated at 37°C, under a humidified, 95 per cent air-5 per cent carbon dioxide atmosphere.

For normal calcium-concentration epidermal cultures, the same method was employed except that the S-MEM medium was supplemented with sterile calcium chloride, such that the final calcium concentration of the complete medium was 1.4 mM (Appendix 5).

After 24 hours, the culture medium was removed from each well and replaced with 200 μl of appropriate supernatant.

MONONUCLEAR CELL CULTURE SUPERNATANTS

The supernatants from SMC cultures, derived from either *D.congolensis*-infected or naive rats, were prepared as previously described (p.108). *D.congolensis*-stimulated (test) and control supernatants, both dialysed and non-dialysed, were examined for their effect on epidermal cell proliferation. The supernatants were diluted with complete S-MEM, low or normal calcium as appropriate, to give 1/2, 1/10, 1/100 and 1/1,000 of the initial concentration. In one experiment, a dilution of 1/10,000 was also tested. The volume of added medium required to achieve the final dilution, was slightly less for the control as compared with the stimulated supernatant because the control supernatants had previously been diluted as a consequence of the reconstitution with *D.congolensis* at the end of the culture period (p.108). After the addition of the supernatants, the epidermal cultures were returned to normal culture conditions.

To determine whether any effects of the supernatants were due to the presence of *D.congolensis* products, supernatants were also prepared from culture medium containing *D.congolensis* at the same concentration as previously; the method was the same as that used for SMC cultures except that SMC were not present. The resultant *D.congolensis* supernatants, which were not dialysed, were then added to the epidermal cultures as for the SMC supernatants.

DETERMINATION OF EPIDERMAL CELL PROLIFERATION RATE

At the appropriate times, 0.5 μCi ^{3}H-thymidine in 50 μl complete medium was added to each well, to give a final concentration of 2 μCi ml^{-1} and the cultures incubated for four hours under the same conditions as before. The cells were harvested, 0, 24 or 48 hours after the addition of

the supernatant. The method of harvesting and determination of incorporated ^{3}H-thymidine has been described (p.130).

ANALYSIS OF RESULTS

Two approaches were taken in analysing the data. The first was to compare the effect of the various supernatants with that of normal culture medium on epidermal cell growth, as assessed by the ^{3}H-thymidine incorporation rate. Whereby:

$$SI = Md_s / Md_m \qquad (3)$$

Where SI is the stimulation index, Md_s is the median CPM of ten replicate cultures incubated with SMC supernatant and Md_m is the median CPM of ten replicate cultures incubated with medium alone.

For reasons which will be discussed later, a more applicable approach to analysing the data, was to compare the effect on epidermal growth of the test SMC supernatants with that of the corresponding control supernatants from unstimulated SMC cultures. Whereby:

$$SI = Md_{ss} / Md_{cs} \qquad (4)$$

Where SI is the stimulation index, Md_{ss} is the median CPM of ten replicate cultures incubated with test SMC supernatant and Md_{cs} is the median CPM of ten replicate cultures incubated with control SMC supernatant.

RESULTS

EFFECT OF SMC SUPERNATANTS ON EPIDERMAL CELL GROWTH

Twenty-four hours after initiation of the epidermal cell cultures little growth had occurred and the uptake of ^{3}H-thymidine was minimal, ranging from 50-500 CPM within the ten experiments conducted. At this time, designated "time 0", the culture medium was replaced with SMC supernatant. By day two of culture, 24 hours after the addition of supernatant, the cultures were growing well, both in culture medium alone and in the presence of supernatant (Figure 6.1). The increase in growth was reflected by the high ^{3}H-thymidine incorporation rate of up to 27,000 CPM, with the value depending on the experiment and the dilution and type of supernatant. An exception was the growth rate in the presence of the lowest dilution of dialysed supernatant, either test or control. Epidermal cell growth was consistently inhibited (Figure 6.2) with ^{3}H-thymidine counts often at background levels and always below that of cells in medium alone (20-2,000 CPM). By day three of culture, 48 hours after addition of supernatants, the cultures were still subconfluent but often possessed small foci of confluence, both in medium alone and in the presence of supernatants. ^{3}H-thymidine incorporation rates ranged from 5,000 to 40,000 CPM. In contrast, epidermal cells incubated with the dialysed supernatants at the lowest dilution, appeared to be growing poorly compared to the other cultures, or not at all, with corresponding ^{3}H-thymidine counts of 20-3,000 CPM.

The ^{3}H-thymidine incorporation rates of the epidermal cell cultures varied with the type and dilution of supernatant. The median CPM values for each of the ten experiments described are presented in appendix five. For each of the ten experiments, the supernatants caused either

stimulation or inhibition of epidermal growth as compared with that in medium alone (Equation 3), depending on the dilution of the supernatant. Typically, the non-dialysed supernatants, either *D.congolensis*-stimulated (test) or control, caused strong stimulation of growth (Mann-Whitney $U^{1}_{10}=16$, $P < 0.01$), with the effect diminishing as the supernatant was diluted out (Figures 6.3 and 6.4). However, for the test supernatant this effect was only noticeable at 48 hours. Supernatants which had been dialysed, deriving from the same SMC cultures as the non-dialysed preparations, caused inhibition of epidermal growth at the lower dilutions but as the dilution factor increased, the effect shifted to one of significant stimulation of growth (Mann-Whitney $U^{1}_{10}=16$, $P < 0.01$, figures 6.3 and 6.4).

EFFECT OF *D.CONGOLENSIS* CULTURE PRODUCTS ON EPIDERMAL GROWTH

The supernatants which derived from cultures containing *D.congolensis* but no SMC, caused significant inhibition of epidermal growth compared with that in medium alone (Mann-Whitney $U^{1}_{10}=16$, $P < 0.01$), with corresponding stimulation indices of between 0.46 and 0.78.

EFFECT OF SMC SUPERNATANTS DERIVED FROM INFECTED RATS ON EPIDERMAL CELL PROLIFERATION RATES IN LOW CALCIUM MEDIUM[+]

At the low dilutions of SMC supernatants, epidermal cells incorporated significantly less ^{3}H-thymidine with test supernatants than with control supernatants, regardless of whether the supernatant was dialysed or not and of the time of assay (Figures 6.5, 6.6 and 6.7, Mann-Whitney $U^{1}_{10}=16$, $P < 0.01$).

[+]Henceforth results are expressed as the effect of the *D.congolensis*-stimulated (test) supernatant compared with that of the control supernatant (Equation 4).

At the higher dilutions, the test supernatants caused significant stimulation of epidermal proliferation at 24 hours (Mann-Whitney $U'^{8}_{8}=16$, $P < 0.01$); with dialysis enhancing this stimulation (Figures 6.5 A, 6.6 A and 6.7 A). By 48 hours, the stimulatory capacity of the higher dilutions of dialysed supernatant had disappeared (Figures 6.5 B, 6.6 B and 6.7 B).

The experiment was carried out three times, with the same pattern of response in each (Figure 6.5, 6.6 and 6.7).

EFFECT OF SMC SUPERNATANTS DERIVED FROM NAIVE RATS ON EPIDERMAL CELL PROLIFERATION RATES IN LOW CALCIUM MEDIUM

At the low dilutions of supernatants derived from naive rats, epidermal proliferation was significantly inhibited by the test compared with the control supernatant (Figures 6.8, 6.9 and 6.10, Mann-Whitney $U'^{8}_{8}=16$, $P < 0.01$). One exception occurred; this was the lowest dilution of dialysed supernatant in one of the repeat experiments, which had no effect on the proliferation rate (Figure 6.9).

The higher dilutions of supernatants derived from naive rats were also not stimulatory for epidermal cell proliferation, unlike those from infected rats. None of the dialysed supernatants caused any significant effect, neither stimulation nor inhibition (Figures 6.8, 6.9 and 6.10). The higher dilutions of the non-dialysed supernatants either had no effect, caused inhibition, or, in one of the three experiments, caused slight stimulation (Figures 6.8, 6.9 and 6.10).

EFFECT OF SMC SUPERNATANTS DERIVED FROM INFECTED RATS ON EPIDERMAL CELL PROLIFERATION RATES IN NORMAL CALCIUM MEDIUM

In the first experiment, the non-dialysed test supernatant derived from infected rats had similar effects on epidermal cell proliferation as did the test supernatant on cells in low calcium concentration-medium. The lower dilutions caused significant inhibition (Mann-Whitney $U^{16}_{16}=16$, $P < 0.01$), but the effect was diluted out although none of the dilutions tested caused stimulation (Figure 6.11). In the second experiment, a repeat of the first, the non-dialysed supernatant had no effect, except at the highest dilution which caused slight but significant stimulation (Figure 6.12, Mann-Whitney $U^{16}_{16}=16$, $P < 0.01$).

The effect of the dialysed test supernatants on epidermal proliferation was similar to that on growth in low calcium medium, in terms of dose-dependency. However, the pattern was reversed, since the lowest dilutions of supernatant had a strong stimulatory effect on epidermal proliferation (Mann-Whitney $U^{16}_{16}=16$, $P < 0.01$), whereas the highest dilutions had no effect (Figures 6.11 and 6.12).

EFFECT OF SMC SUPERNATANTS DERIVED FROM NAIVE RATS ON EPIDERMAL CELL PROLIFERATION RATES IN NORMAL CALCIUM MEDIUM

In general, the test supernatants from naive rats had a similar effect on epidermal cells in normal, as in low calcium-concentration medium. The lower dilutions caused significant inhibition of proliferation (Mann-Whitney $U^{16}_{16}=16$, $P < 0.01$), whereas at the higher dilutions little effect was noticeable (Figures 6.13 and 6.14). In one experiment, slight but significant stimulation occurred at one dilution (Figure 6.13).

DISCUSSION

Taken as a whole, the results suggest the presence of several factors in the supernatants, some of which stimulate and some of which inhibit the growth of cultured epidermal cells. Both dialysable and non-dialysable activities were present, suggesting variable molecular weight entities, both below and above 10,000 daltons. One or more factors only became apparent in their effect when other activities had been diluted out.

The predominant effect of non-dialysed test and control supernatants was to cause significant stimulation of epidermal proliferation compared with that in medium alone (Figures 6.3 and 6.4). The stimulation was particularly strong at the lower dilutions and was likely to be due, at least partly, to the well-recognised phenomenon of conditioned-medium growth-enhancement (Ling and Kay 1975). Another possibility was that the RPMI medium itself was more favourable to epidermal cell growth than SMEM. Dialysis of the supernatants appeared to remove all of the non-specific stimulation apparent at the lower dilutions, with resultant growth inhibition by both test and control supernatants. At the higher dilutions, both test and control supernatants caused significant stimulation of growth compared with medium alone, with the effect apparent at different times (Figure 6.3 and 6.4). These general stimulatory and inhibitory effects may have been due to small molecular weight by-products of SMC metabolism. Some of the inhibitory substances were likely to be products released from dying SMC.

Thus, SMC supernatants affect *in vitro* epidermal growth, regardless of whether derived from antigen-stimulated or control cultures. To determine effects which were specific to *D.congolensis*-stimulated SMC, it was

necessary to compare these with the control supernatants, expressing the result as the stimulation index.

The *D.congolensis*-stimulated supernatants derived from infected rats appeared to contain a preponderance of inhibitory factors for epidermal cells cultured in low calcium-concentration medium. Stimulatory factors were also present but only became apparent when the effect of the inhibitory substances had been diluted out. This may reflect different cell surface receptor affinities for the various factors, such that the stimulatory factor(s) is recognised by a receptor with a higher affinity than that of the receptor(s) for the inhibitory factor(s). Some of the inhibitory factors were removed from the supernatants by dialysis, hence the enhancement of stimulation, suggesting that these were small molecular weight products. Likewise, Krueger and Jederberg (1980) found that mononuclear cell supernatants were generally inhibitory to HeLa cell proliferation but that dialysis removed some of the inhibition.

When the same type of supernatants were tested on epidermal cells cultured in normal calcium-concentration medium, both stimulatory and inhibitory activities were again identified. However, a different pattern of response was observed from that in low calcium medium. Dialysis of the supernatants removed virtually all of the inhibitory activity observed at the low dilutions. Nevertheless, some inhibitory factors may still have been present but their effect swamped by the stimulatory factors. Stimulation of epidermal growth in normal calcium medium only occurred with the lower dilutions of supernatant.

There are several possible reasons for the different pattern of effects on low and normal calcium cultures. The cells may have been responding to different growth-stimulatory and inhibitory factors in calcium-deficient medium, as opposed to normal medium; alternatively, they may have been

responding to the same factors, but with different kinetics.

Calcium plays a central role in many growth-related activities. Exogenous calcium is essential for the PHA-induced DNA synthesis by lymphocytes (Whitney and Sutherland 1972). Calcium is required for the binding of PHA to cells but it seems to be the PHA molecule rather than the cell receptor which incorporates the calcium (Kay 1971). Most, if not all, growth factors act via specific receptors on the cell surface and it is possible that expression of certain receptors is calcium-dependent. Extracellular calcium is also essential for the action of almost all hormones (Rasmussen 1970).

Calcium may inhibit as well as stimulate the activity of various factors. Okamoto and Mayer (1978) found that a calcium concentration of 1-2 mM, which is equivalent to that of the normal calcium epidermal cultures, inhibited the action of lymphotoxin. This lymphokine appears to be either growth-inhibitory or cytotoxic depending on the concentration and is probably identical to proliferation inhibitory factor and colony inhibitory factor (Yoshida 1979).

Different response times are characteristic of various classes of growth factor (Shipley 1986). This may explain why some effects were observed at 24 hours and others at 48 hours after addition of the supernatants; alternatively inherent variability within the assay may have been the cause.

In chapter four it was shown that the supernatants from SMC cultures incubated with *D.congolensis*, which derived from naive rats did not appear to contain macrophage migration inhibition factor. In view of this, the test supernatants from naive rats might not have been expected to cause significantly different effects on epidermal cell proliferation than the control supernatants. Alternatively, since *D.congolensis* does

cause some non-specific stimulation of naive SMC (Chapter 2), the supernatants might have been expected to cause slight stimulation or inhibition, which would be much less than that for infected rats. Indeed, both in low and normal calcium concentration medium, stimulatory effects were usually absent and were weak when they did occur. However, the supernatants caused strong inhibition of proliferation at the lower dilutions. One possibility is that the cultures, from which the supernatants derived, contained more dead and dying SMC than the control due to the toxicity of *D.congolensis* itself for SMC. Released products of these dead SMC may have inhibited epidermal cell proliferation.

The comparison of test and control supernatants in their effect on epidermal proliferation was not interfered with by carried-over products of *D.congolensis* itself because the control supernatants had been reconstituted with an equivalent amount of the *D.congolensis* preparation. Thus, general effects such as those of *D.congolensis* or those due to conditioned or spent medium did not affect the stimulation index results, comparing antigen-stimulated and control supernatants.

To determine whether some of the effects of the supernatants were due to the presence of *D.congolensis*, supernatants were prepared in the same way as for the SMC cultures, except that no mononuclear cells were added. These *D.congolensis* supernatants caused significant inhibition of epidermal growth compared with that in medium alone. In comparison, the same dilution of SMC supernatant caused strong growth stimulation. Thus it seems likely that any *D.congolensis* products in the SMC supernatants only have inhibitory effects and these are swamped by the much stronger stimulatory factors present.

In all the experiments, the cultures were sub-confluent over the assay period. Therefore, under favourable conditions, the cultures would have

been in the exponential phase of growth when the cells should be most susceptible to growth stimulatory or inhibitory factors (Kappler and Marrack 1986). Indeed, Krueger and Jederberg (1980) observed that antigen-stimulated mononuclear cell supernatants only influenced HeLa cell proliferation when the cultures were subconfluent.

Some workers have advocated the use of serum-free medium in growth assays (Gilchrest 1986) but serum was found to be an essential componenent of the rat epidermal culture medium (Chapter 5). In initial experiments, serum was included at the beginning of the cultures and then removed prior to addition of the supernatants under test. The supernatants were also derived from serum-free (SMC) cultures. Although both test and control supernatants produced significant stimulation of epidermal proliferation compared with growth in medium alone (Mann-Whitney $U^{1}_{8}=16$, $P < 0.01$), the overall paucity of growth made the serum-free assay untenable. Thus, the possibility that the effect of growth enhancers might be swamped by serum factors, could not be eliminated. Nevertheless, significant differences in proliferation rates in the presence of various supernatants were demonstrated. Moreover, differences in effect were not due to variable composition of the serum, since all supernatants were diluted in the same batch of medium to ensure uniformity for each experiment.

Lymphokines are known to affect the growth of lymphoid cells, such as the stimulation of activated T-lymphocyte proliferation by interleukin-2 (Roitt *et al* 1985). The effect of lymphokines on the growth of non-lymphoid cells is less well documented. Korszun *et al* (1981) produced PPD-stimulated SMC culture supernatants from guinea-pigs previously immunised with Freunds' complete adjuvant using the same culture methodology as that used in this study. The supernatants, which were

dialysed, caused an increase in the mitotic index of epidermal cells after a small volume had been injected intradermally. The increase caused by the antigen-stimulated supernatant was significantly greater than that caused by the control supernatant and became maximal 24 hours after injection. In association with the stimulation of mitosis a marked acanthosis of the epidermis at the site of injection occurred with the test supernatant. The drawback of this type of study is that it is not possible to assign the observed effects on the epidermis to the direct action of the SMC supernatants. A cellular infiltrate, composed of PMN, lymphocytes and eosinophils collected at the site of injection, so it is possible that these cells were the direct cause of the increased epidermal proliferation.

Antigen-stimulated lymphocyte culture supernatants have been shown to cause significant stimulation of *in vitro* fibroblast proliferation, with a maximal three-fold stimulation by dialysed supernatants at a dilution of one in two (Wahl, Wahl and McCarthy 1978). In contrast, Krueger and Jederberg (1980) found mononuclear cell supernatants were generally inhibitory to HeLa cell proliferation, but supernatants derived from patients suffering from the proliferative skin disease psoriasis were significantly less inhibitory than those derived from normal subjects. Both dialysis and dilution of the supernatants led to varying degrees of inhibition or stimulation and the authors concluded that several factors were present which acted competitively on HeLa cell growth.

Culture supernatants of antigen-stimulated mononuclear cells are likely to contain a wide array of lymphokines (Kappler and Marrack 1986), some of which may affect cell growth. Gamma interferon (IFNγ) is secreted by activated T-lymphocytes (Alm 1987) and, at high concentration, inhibits *in vitro* epidermal cell proliferation (Nickoloff, Basham, Merigan and

Morhenn 1984). Another factor, produced by activated T-cells, which may inhibit epidermal proliferation is lymphotoxin. This lymphokine was mentioned earlier as probably being identical to proliferation inhibition factor.

Interleukin-1 (IL-1) is another candidate for a factor which can regulate epidermal growth. Certain bacterial components, such as peptidoglycans and lipopolysaccharide, induce IL-1 synthesis by monocytes (Dinarello 1984) and IFNγ will augment this production (Arenzana-Seisdedos, Virelizier and Fiers 1985). IL-1 stimulates the growth of fibroblasts (Schmidt, Mizel, Cohen and Green 1982) but reports conflict regarding its effect on epidermal cell growth. Ristow (1987) found that both recombinant IL-1 and monocyte culture supernatants caused cultured mouse epidermal cells to incorporate ^{3}H-thymidine at two to three times the control level, provided that the epidermal cells were "growth-arrested". In contrast, Morhenn (1988) found that IL-1 did not stimulate *in vitro* epidermal proliferation. A possible reason for this anomaly is interference by the presence of IL-1 in epidermal cell cultures due to secretion by epidermal cells themselves. Gilchrest and Sauder (1984) demonstrated that epidermal cells release epidermal cell-derived thymocyte activating factor (ETAF) which stimulates epidermal cell growth ie. acts an autocrine growth factor. ETAF has been shown to be identical to IL-1 (Sauder 1985). The SMC supernatants added to the rat epidermal cultures may well have contained IL-1 secreted by monocytes and IFNγ secreted by T-cells. In addition, the IFNγ may have stimulated the epidermal cells to release IL-1, which, in turn, led to increased cell proliferation.

Another factor which may be present in the supernatants and which might affect epidermal cell proliferation is tumour necrosis factor alpha

(TNFα) which stimulates fibroblast growth (Vilcek, Palombella, Henriksen-Destefano *et al* 1986). TNFα is produced by monocytes and null lymphocytes after stimulation with, for example, the bacterial product lipopolysaccharide (Nedwin, Svedersky, Bringman, Palladino and Goeddel 1985) and is known to induce IL-1 production by certain cells (Alm 1987).

Without purification of the various activities present in the supernatants, it is impossible to know which factors are responsible for the observed effects on epidermal cell proliferation. Nevertheless, it is the overall effect of the supernatant which governs the epidermal cell response; presumably mononuclear cells infiltrating the skin also release a wide range of substances. Factors such as the distance of the mononuclear cells from the epidermal cells and the number of cells present may be important. If released factors have to diffuse a comparatively long distance to reach the basal epidermal cells, then it might only be those which act via high affinity receptors which will have any effect on epidermal proliferation. Conversely, if the factors are released adjacent to the epidermis then the local concentration would be relatively high and even those recognised by low affinity receptors would have an effect.

FIGURE 6.1 Epidermal cells cultured in the presence of dialysed test supernatant at 1/100 dilution for 24 hours.

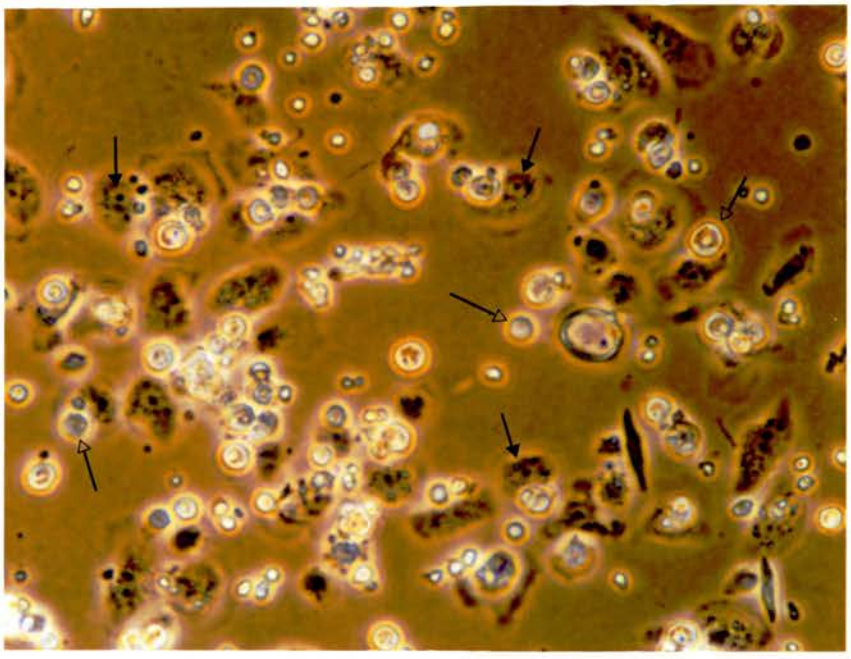
Phase contrast showing many attached, spread cells (closed arrows). Attached rounded-up cells are also present (open arrows).

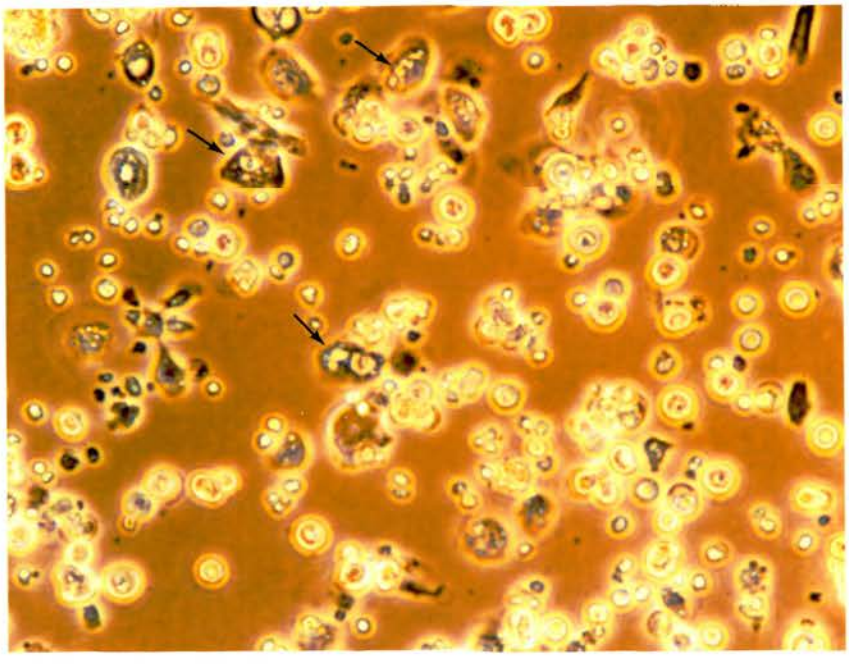
(x 340 magnification)

FIGURE 6.2 Epidermal cells cultured in the presence of dialysed test supernatant at 1/2 dilution for 24 hours.

Phase contrast showing few attached, spread cells; most of these cells are highly vacuolated (arrows).

(x 340 magnification)





EFFECT OF SMC-SUPERNATANTS ON EPIDERMAL PROLIFERATION RATES

FIGURE 6.3: Effect of test supernatant on epidermal proliferation at A) 24 hours
B) 48 hours

FIGURE 6.4: Effect of control supernatant on epidermal proliferation at A) 24 hours
B) 48 hours

Histograms show stimulation indices with different dilutions of supernatant. The indices derive from comparison of the effect of supernatant with that of medium alone on epidermal proliferation (Equation 3, page 163).

Striped bars represent non-dialysed supernatant

Hatched bars represent dialysed supernatant

All values above one represent significant stimulation of proliferation and below one significant inhibition of proliferation (Mann-Whitney, $U^{10}_{10}=16$, $P < 0.01$), unless otherwise indicated (NS).

Note that the statistical significance refers to the stimulation/inhibition by each supernatant and not to differences between dialysed and non-dialysed supernatants.

Epidermal cultures were incubated in low calcium medium.

Figure 6.3A

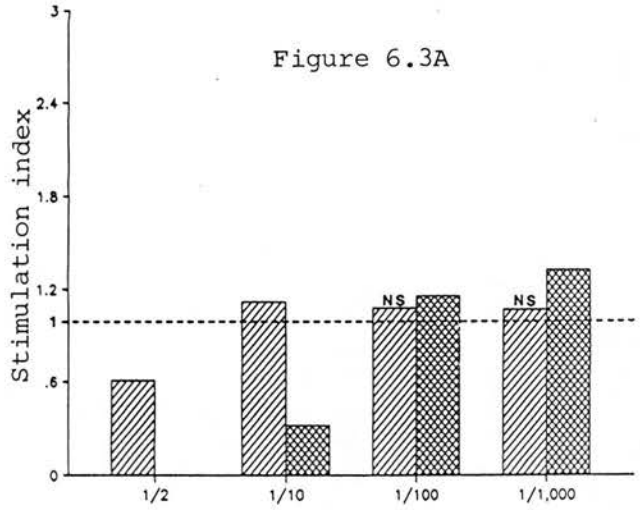


Figure 6.3B

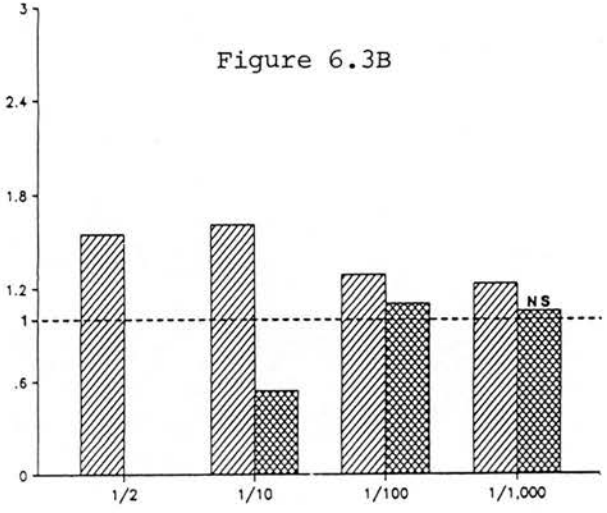


Figure 6.4A

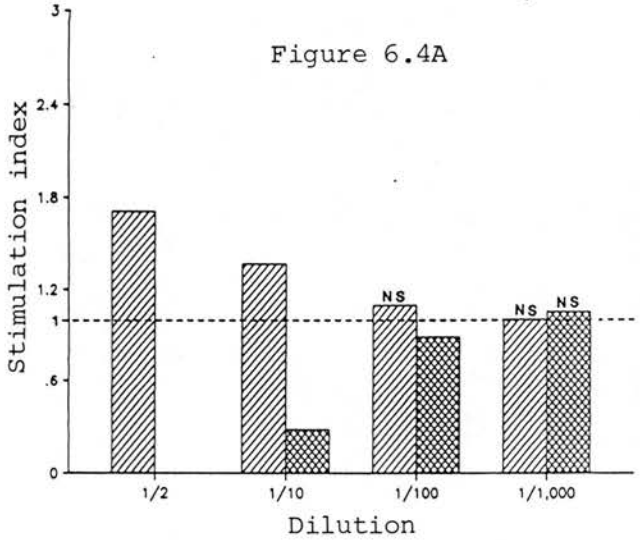
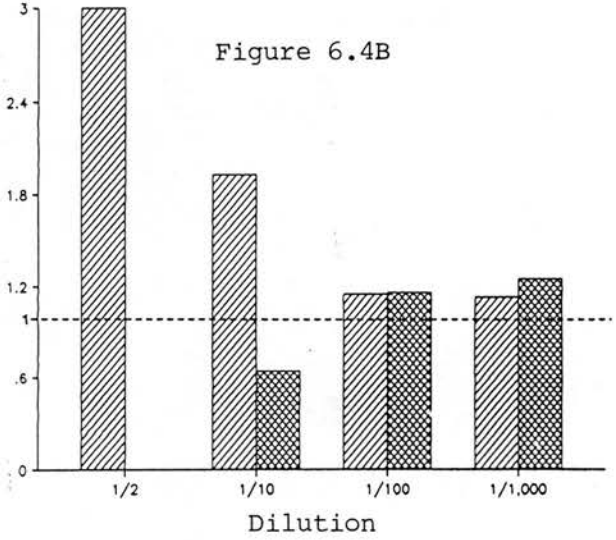


Figure 6.4B



EFFECT OF SMC-SUPERNATANTS DERIVED FROM INFECTED RATS ON EPIDERMAL PROLIFERATION RATES IN LOW CALCIUM MEDIUM

FIGURE 6.5: Experiment one A) 24 hours
B) 48 hours
FIGURE 6.6: Experiment two A) 24 hours
B) 48 hours
FIGURE 6.7: Experiment three A) 24 hours
B) 48 hours

Experiments two and three are repeats of experiment one

Histograms show stimulation indices with different dilutions of supernatant. The indices derive from the comparison of *D.congolensis*-stimulated supernatant and control supernatant (Equation 4, page 163).

Striped bars represent non-dialysed supernatant

Hatched bars represent dialysed supernatant

All values above one represent significant stimulation of proliferation and below one significant inhibition of proliferation (Mann-Whitney, $U'^{0.01}_{10}=16$, $P < 0.01$), unless otherwise indicated (NS).

Note that the statistical significance refers to the stimulation/inhibition by each supernatant and not to differences between dialysed and non-dialysed supernatants.

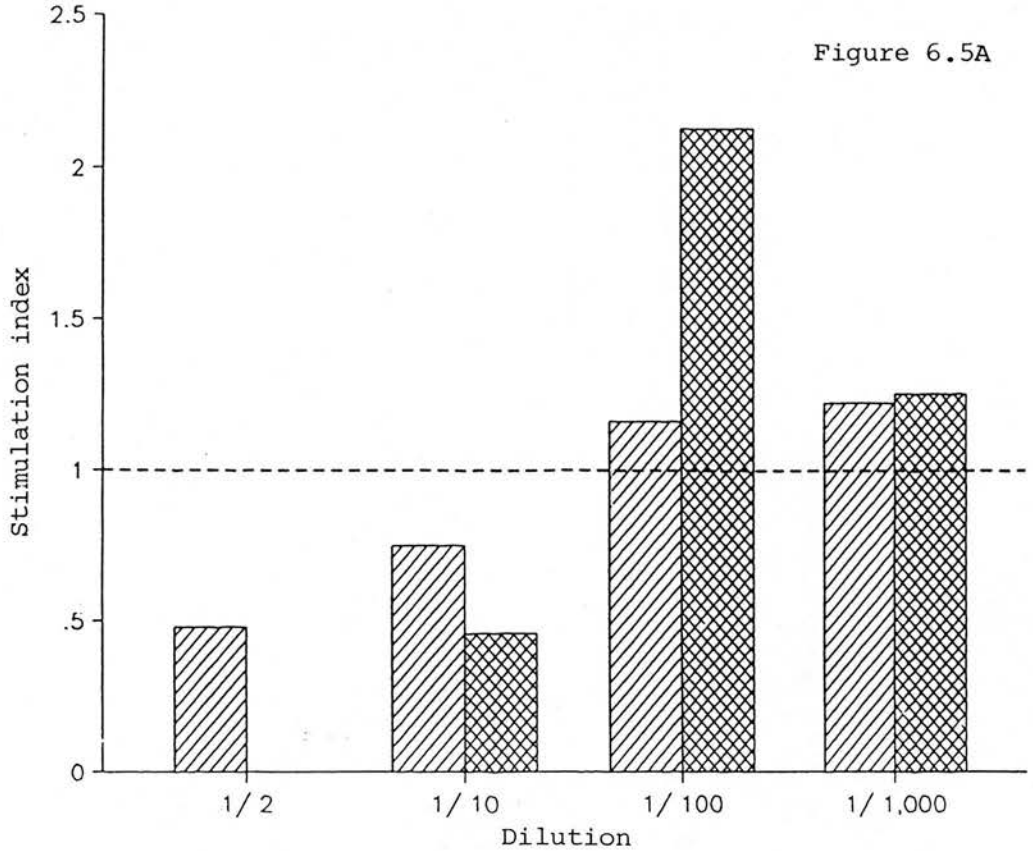


Figure 6.5A

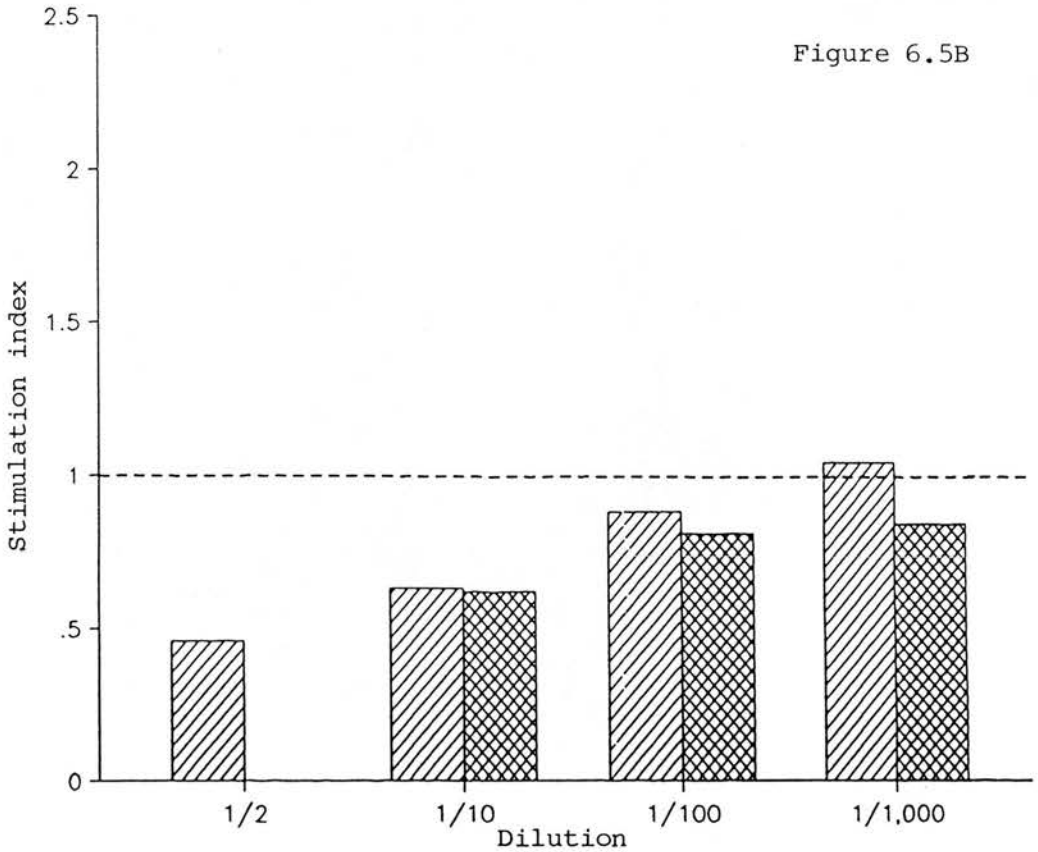


Figure 6.5B

Figure 6.6A

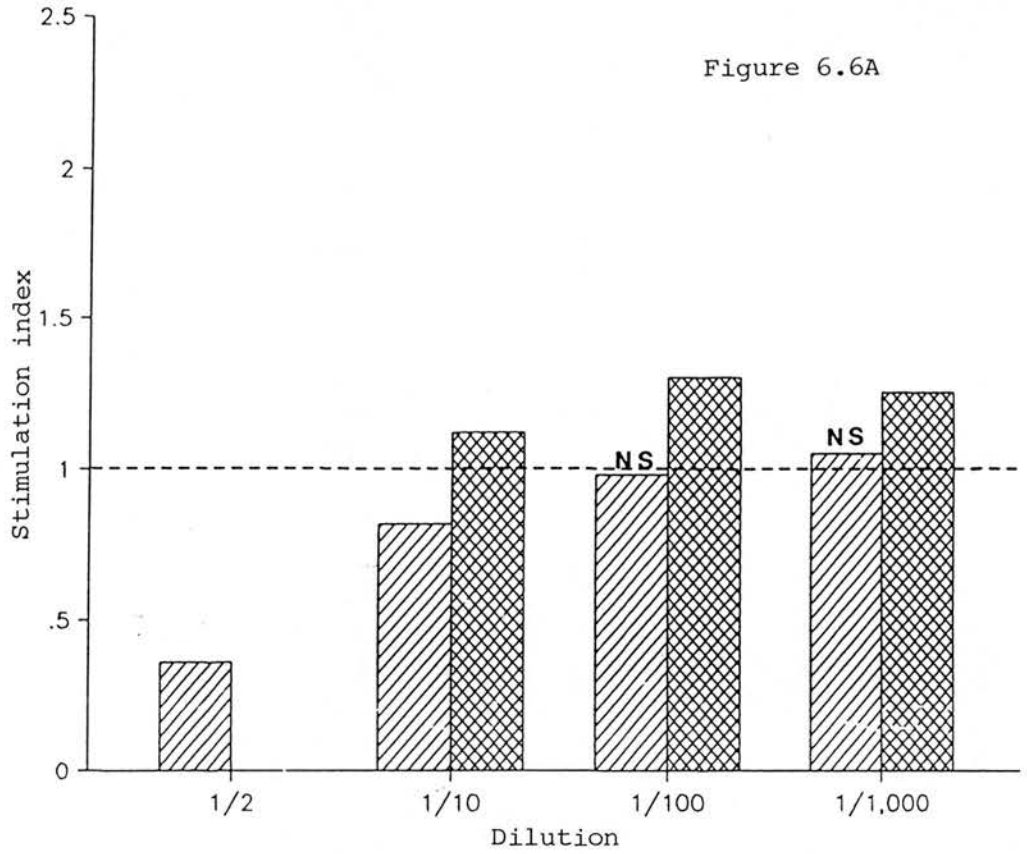
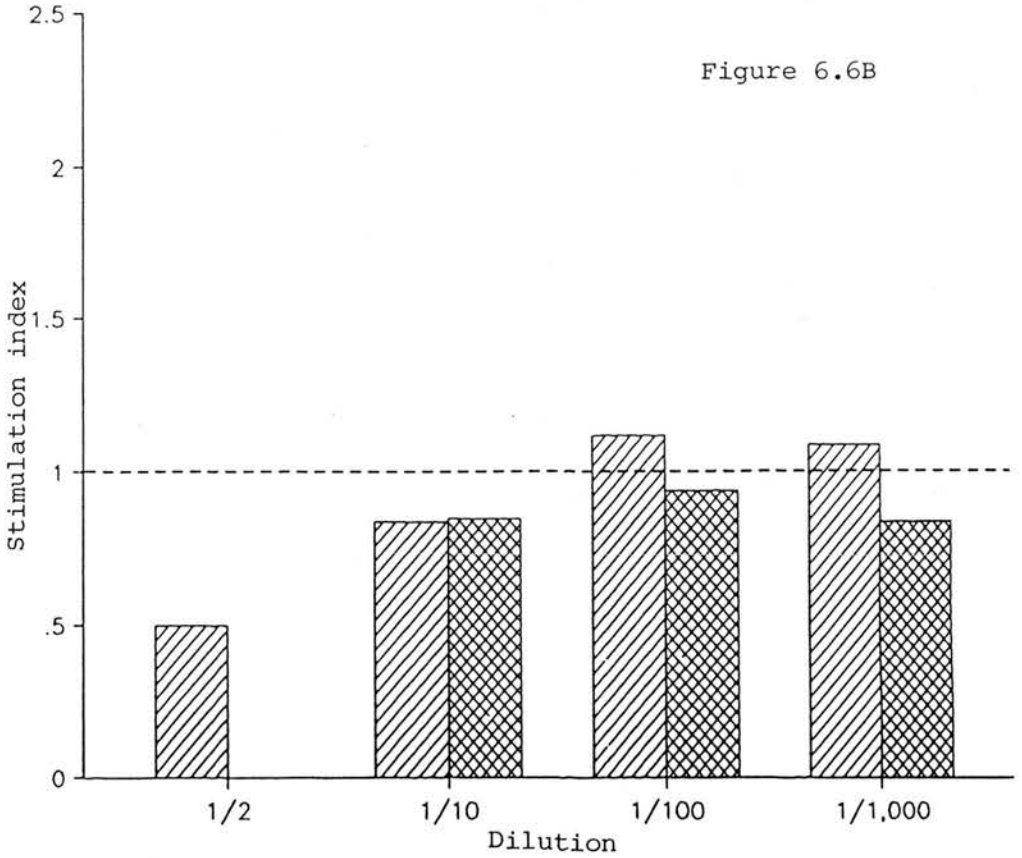


Figure 6.6B



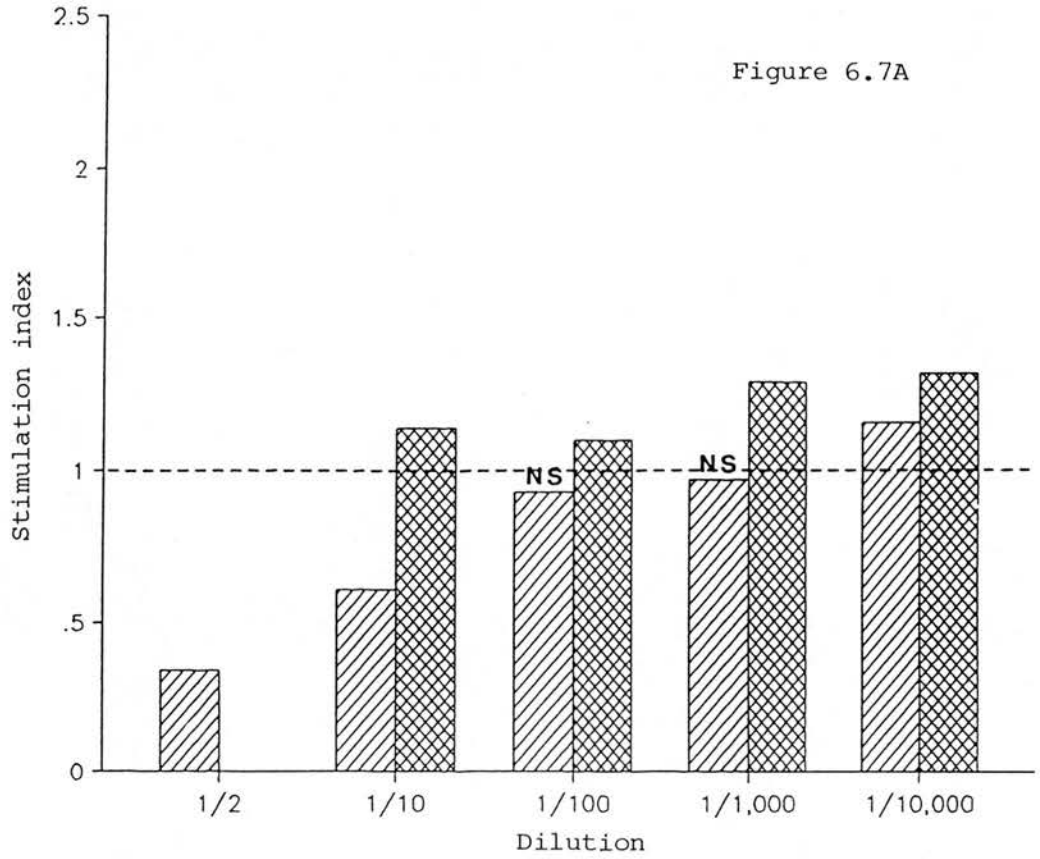


Figure 6.7A

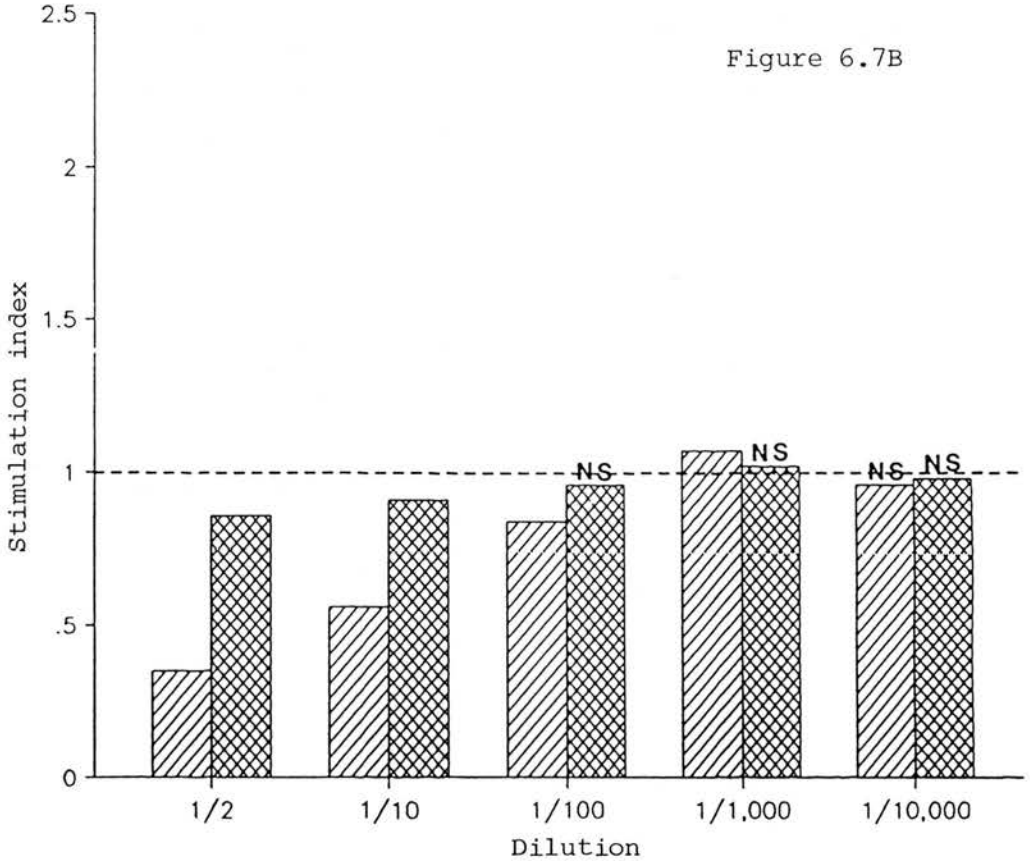


Figure 6.7B

EFFECT OF SMC-SUPERNATANTS DERIVED FROM NAIVE RATS ON EPIDERMAL PROLIFERATION RATES IN LOW CALCIUM MEDIUM

FIGURE 6.8: Experiment four A) 24 hours
B) 48 hours
FIGURE 6.9: Experiment five A) 24 hours
B) 48 hours
FIGURE 6.10: Experiment six A) 24 hours
B) 48 hours

Experiments five and six are repeats of experiment four

Histograms show stimulation indices with different dilutions of supernatant. The indices derive from the comparison of *D.congolensis*-stimulated supernatant and control supernatant (Equation 4, page 163).

Striped bars represent non-dialysed supernatant

Hatched bars represent dialysed supernatant

All values above one represent significant stimulation of proliferation and below one significant inhibition of proliferation (Mann-Whitney, $U^{10}_{10}=16$, $P < 0.01$), unless otherwise indicated (NS).

Note that the statistical significance refers to the stimulation/inhibition by each supernatant and not to differences between dialysed and non-dialysed supernatants.

Figure 6.8A

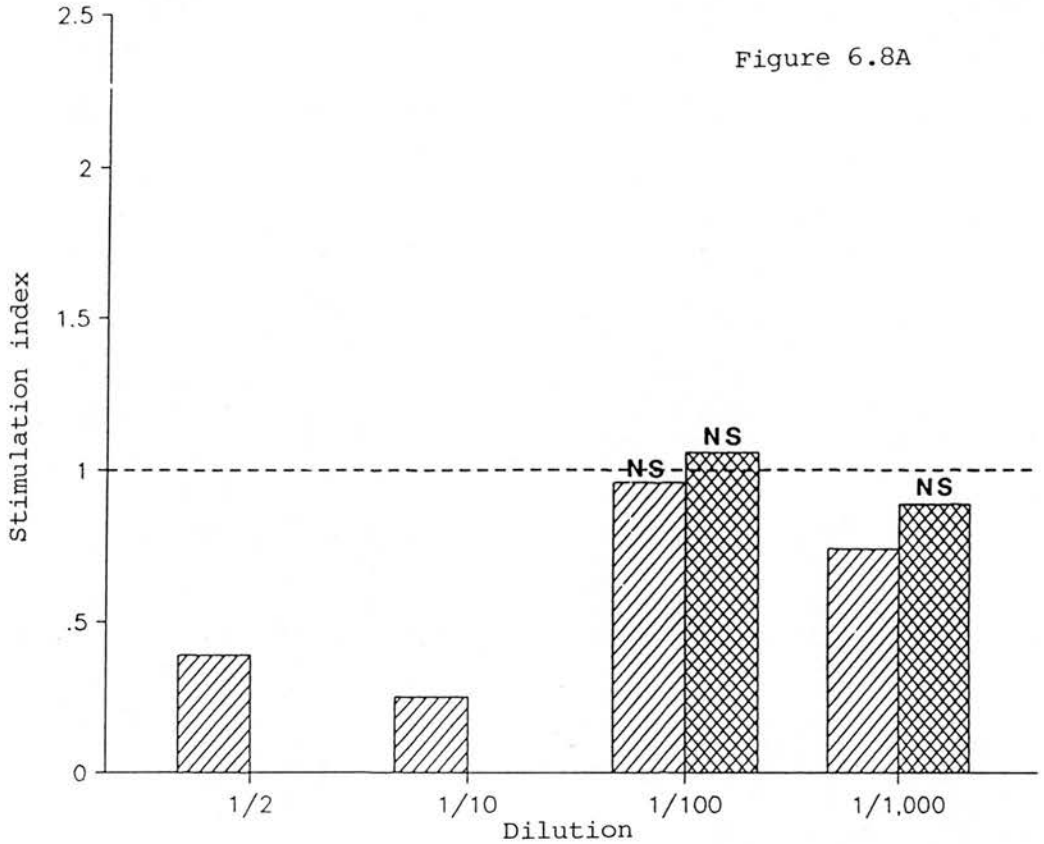
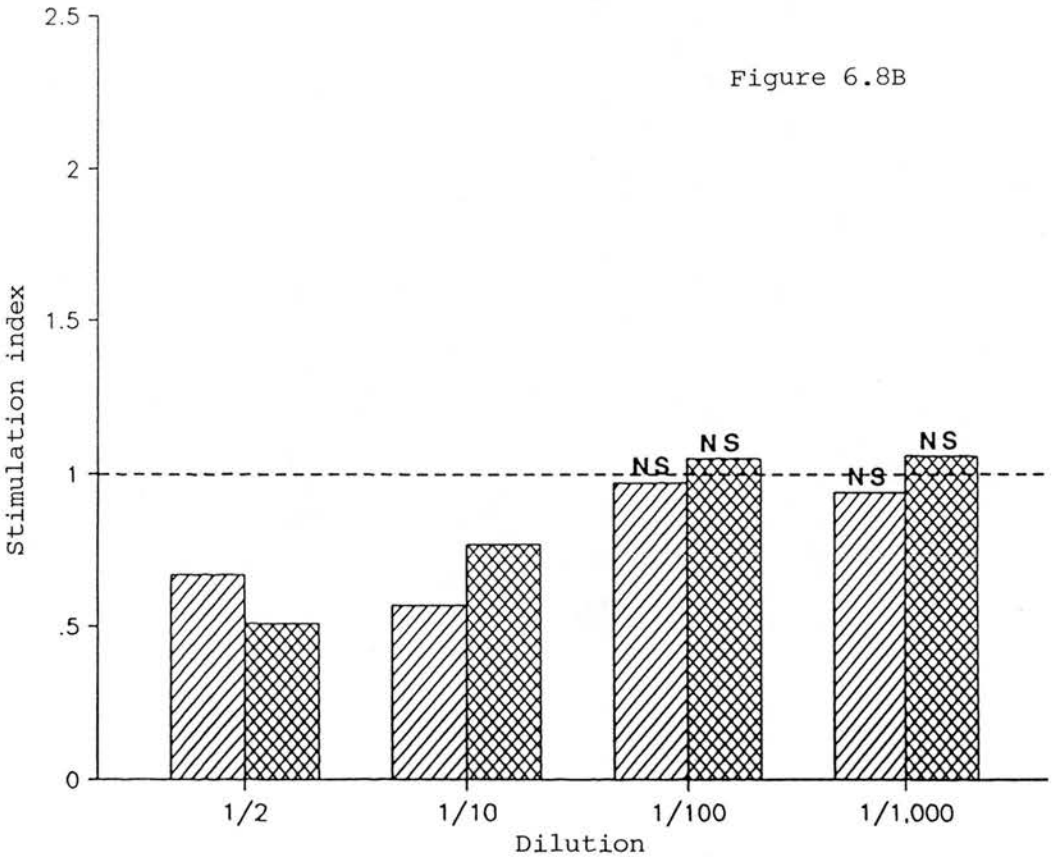


Figure 6.8B



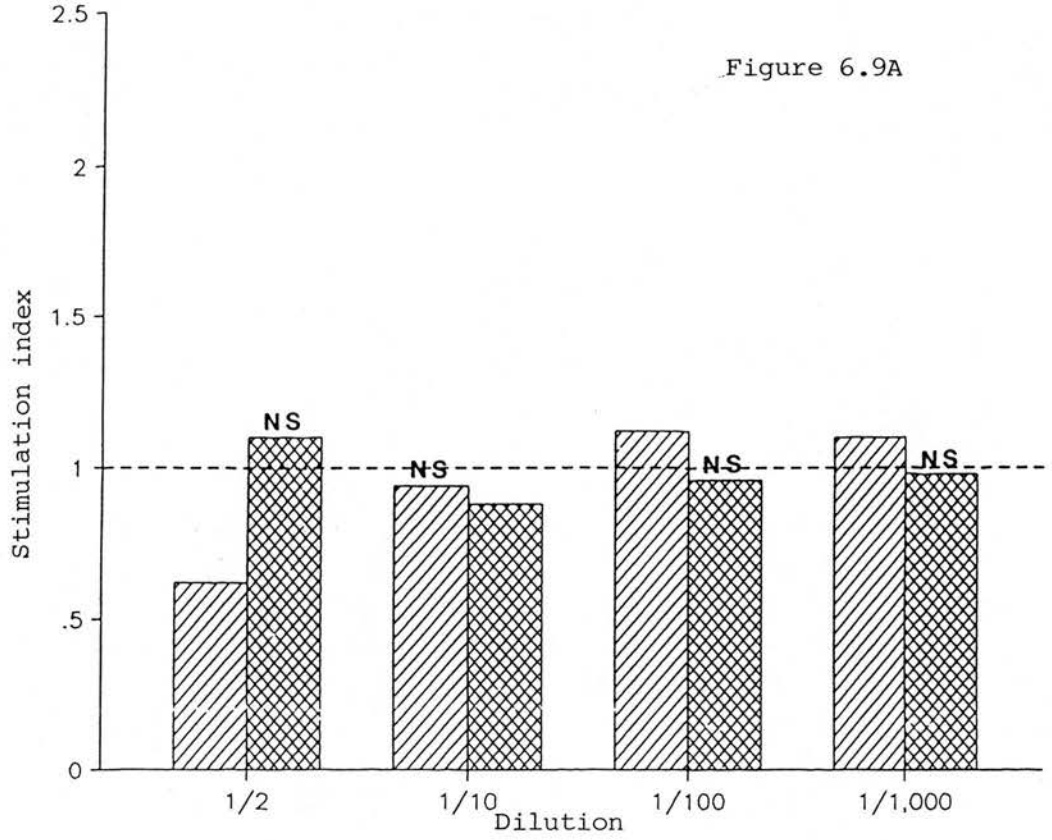


Figure 6.9A

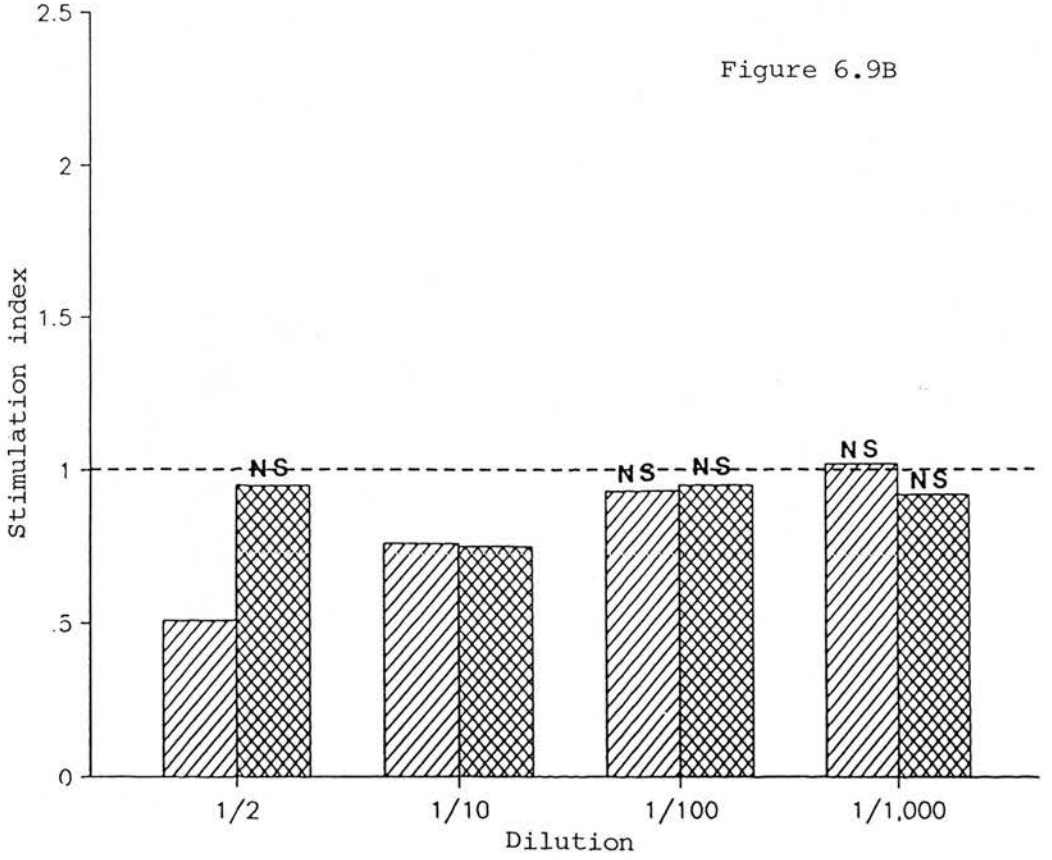


Figure 6.9B

Figure 6.10A

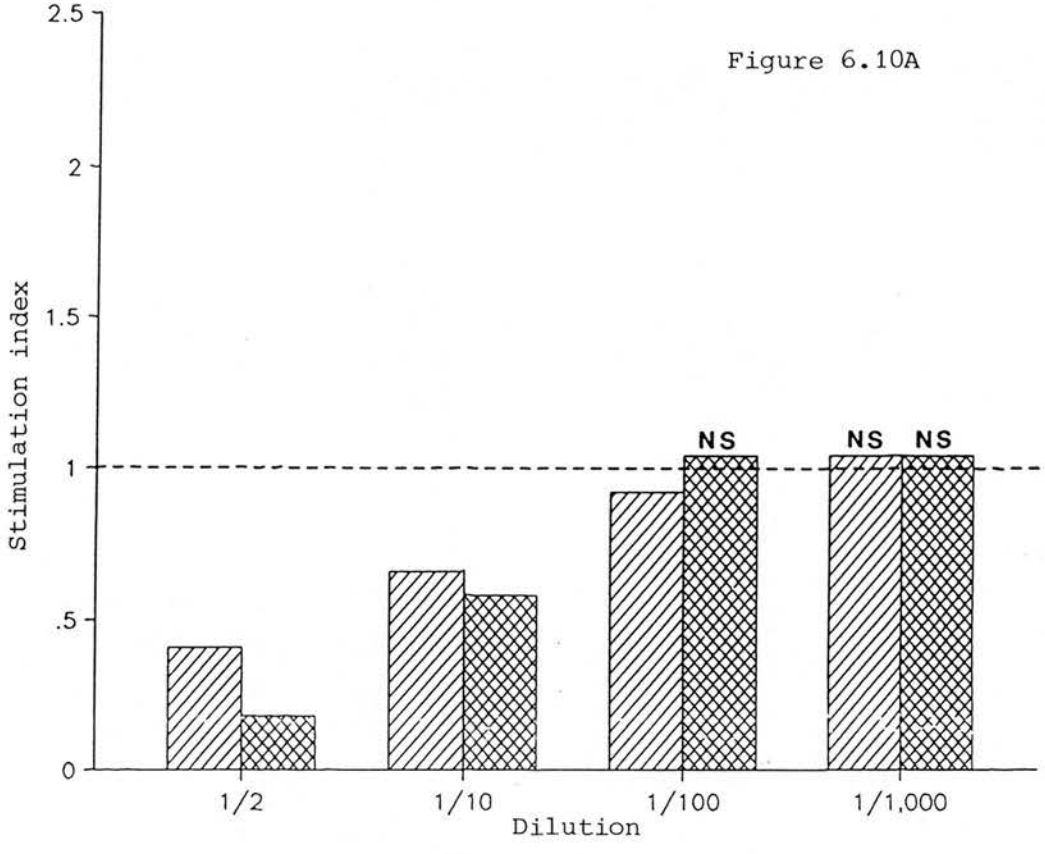
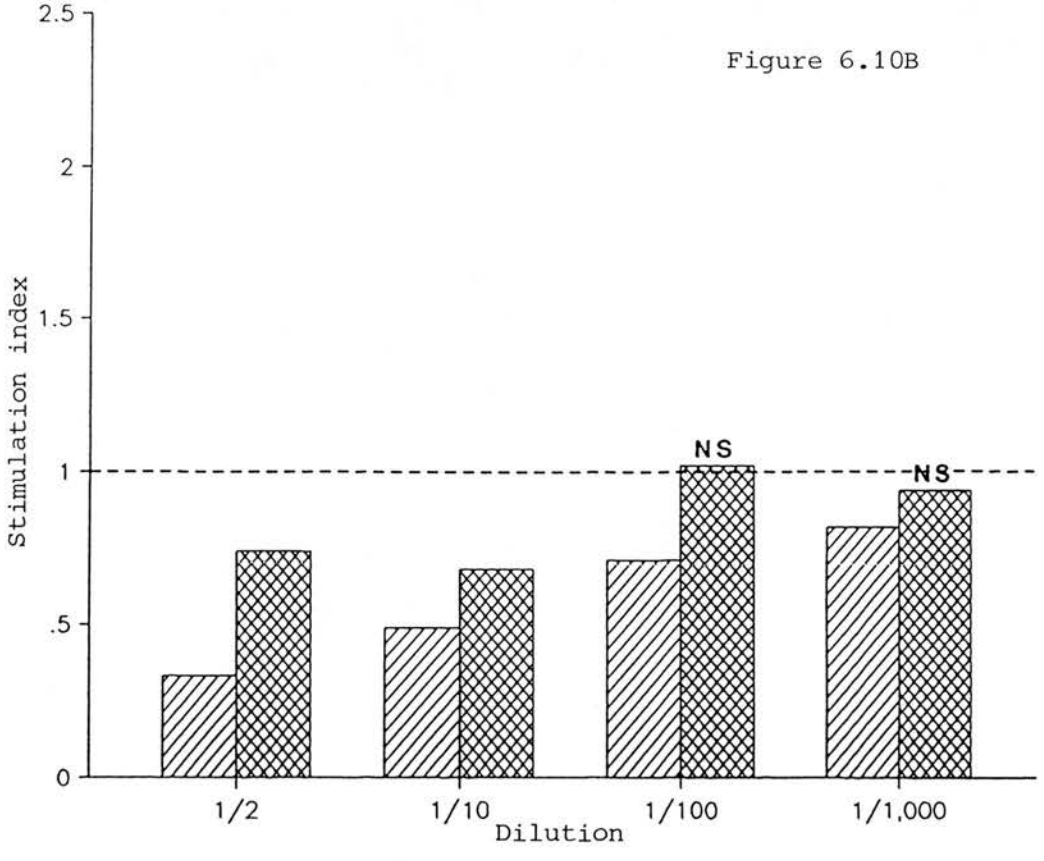


Figure 6.10B



EFFECT OF SMC-SUPERNATANTS DERIVED FROM INFECTED RATS ON EPIDERMAL PROLIFERATION RATES IN NORMAL CALCIUM MEDIUM

FIGURE 6.11: Experiment seven A) 24 hours
B) 48 hours
FIGURE 6.12: Experiment eight A) 24 hours
B) 48 hours

Experiment eight was a repeat of experiment seven.

Histograms show stimulation indices with different dilutions of supernatant. The indices derive from the comparison of *D.congolensis*-stimulated supernatant and control supernatant (Equation 4, page 163).

Striped bars represent non-dialysed supernatant

Hatched bars represent dialysed supernatant

All values above one represent significant stimulation of proliferation and below one significant inhibition of proliferation (Mann-Whitney, $U^{16}_{16}=16$, $P < 0.01$), unless otherwise indicated (NS).

Note that the statistical significance refers to the stimulation/inhibition by each supernatant and not to differences between dialysed and non-dialysed supernatants.

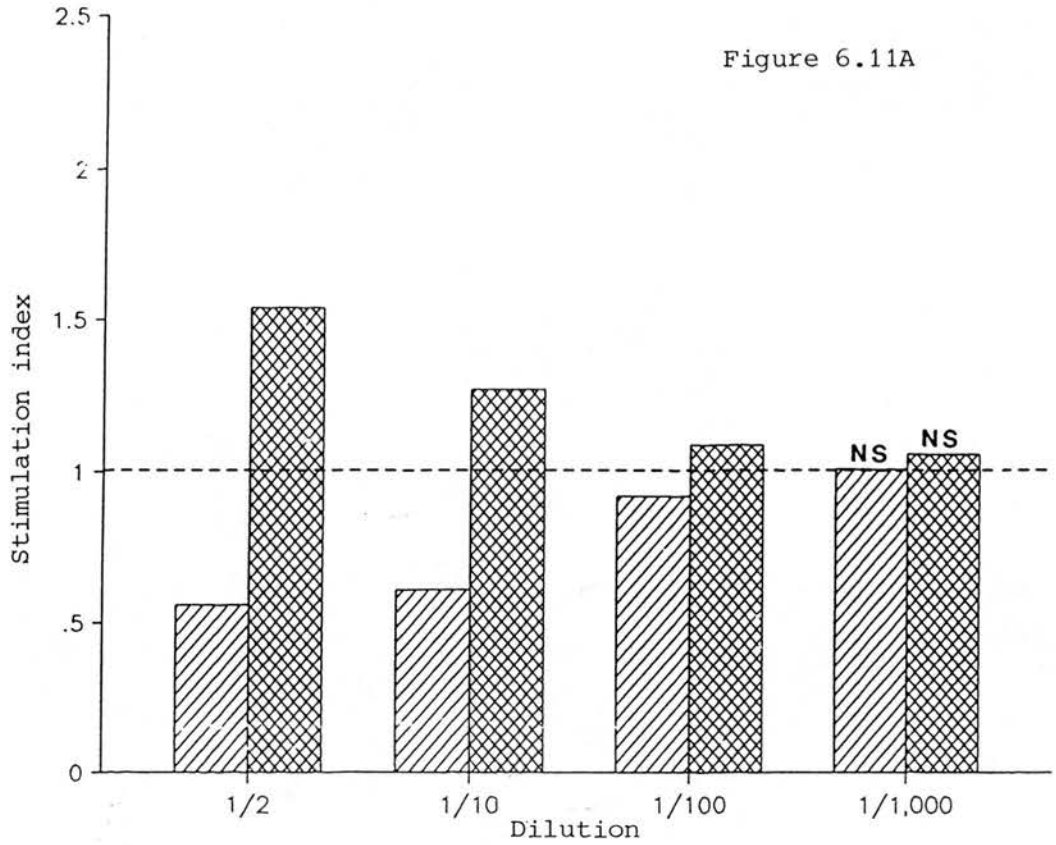


Figure 6.11A

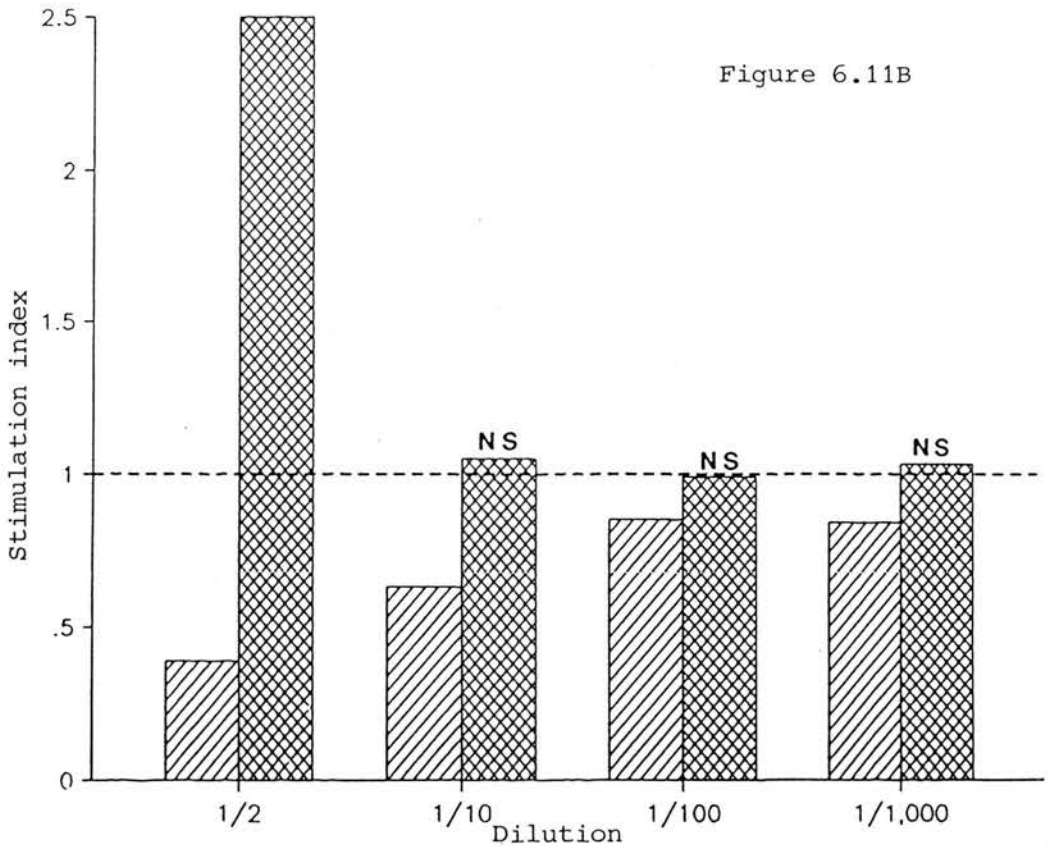


Figure 6.11B

Figure 6.12A

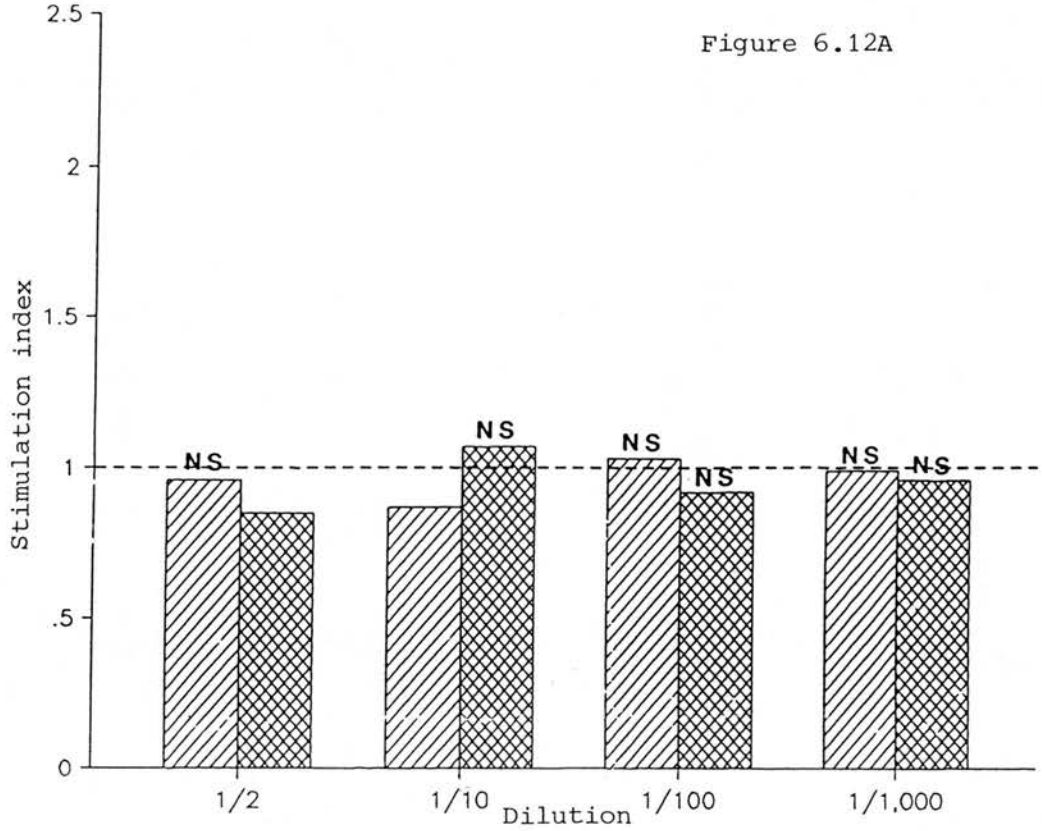
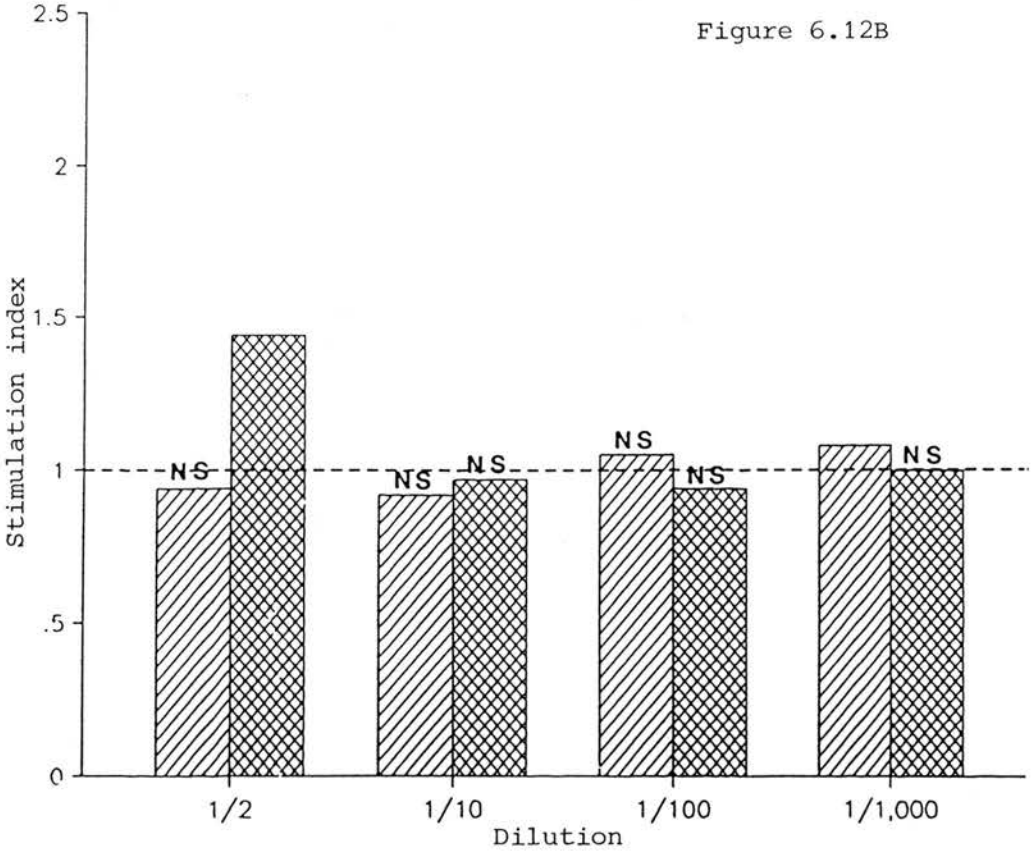


Figure 6.12B



EFFECT OF SMC-SUPERNATANTS DERIVED FROM NAIVE RATS ON EPIDERMAL PROLIFERATION RATES IN NORMAL CALCIUM MEDIUM

FIGURE 6.13: Experiment nine A) 24 hours
B) 48 hours
FIGURE 6.14: Experiment ten A) 24 hours
B) 48 hours

Experiment ten was a repeat of experiment nine.

Histograms show stimulation indices with different dilutions of supernatant. The indices derive from the comparison of *D.congolensis*-stimulated supernatant and control supernatant (Equation 4, page 163).

Striped bars represent non-dialysed supernatant

Hatched bars represent dialysed supernatant

All values above one represent significant stimulation of proliferation and below one significant inhibition of proliferation (Mann-Whitney, $U^{10}_{10}=16$, $P < 0.01$), unless otherwise indicated (NS).

Note that the statistical significance refers to the stimulation/inhibition by each supernatant and not to differences between dialysed and non-dialysed supernatants.

Figure 6.13A

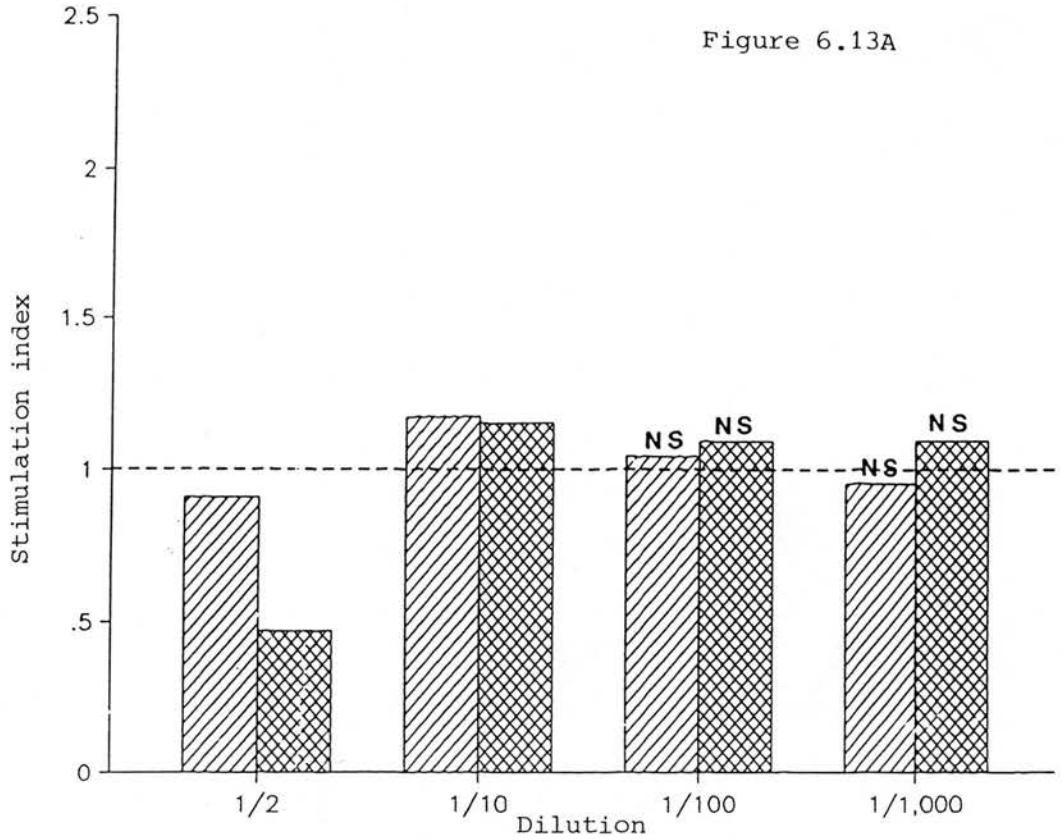


Figure 6.13B

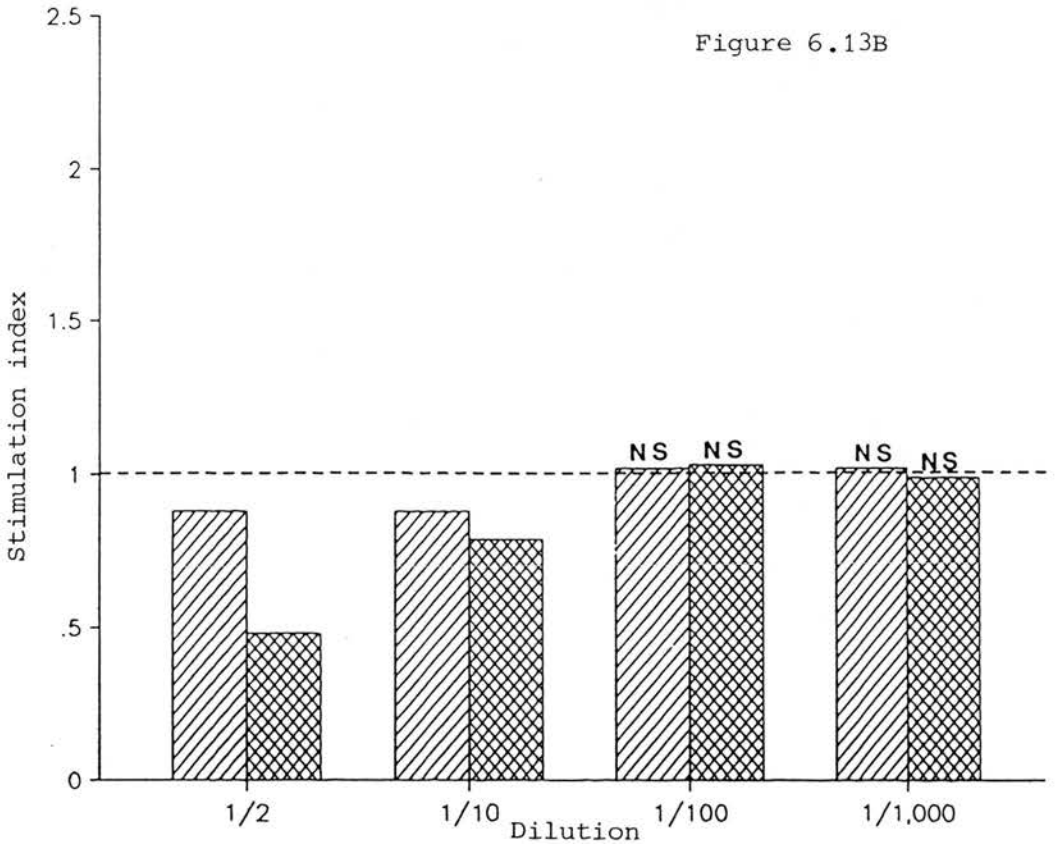


Figure 6.14A

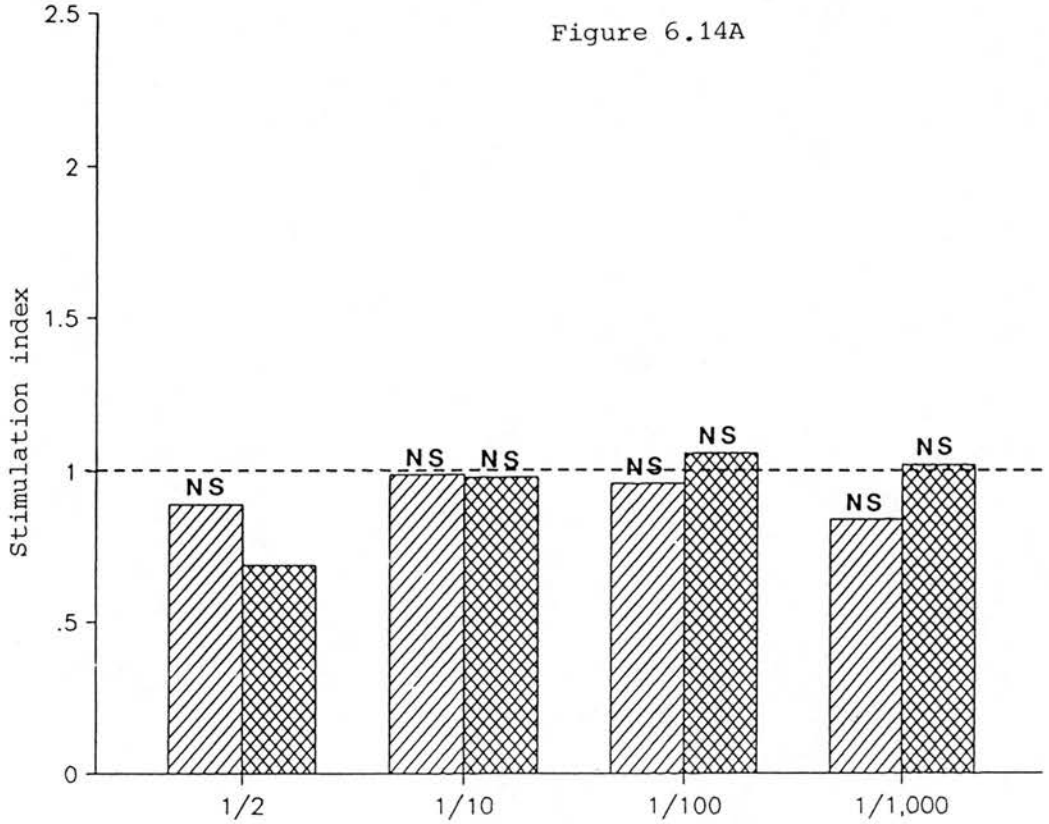
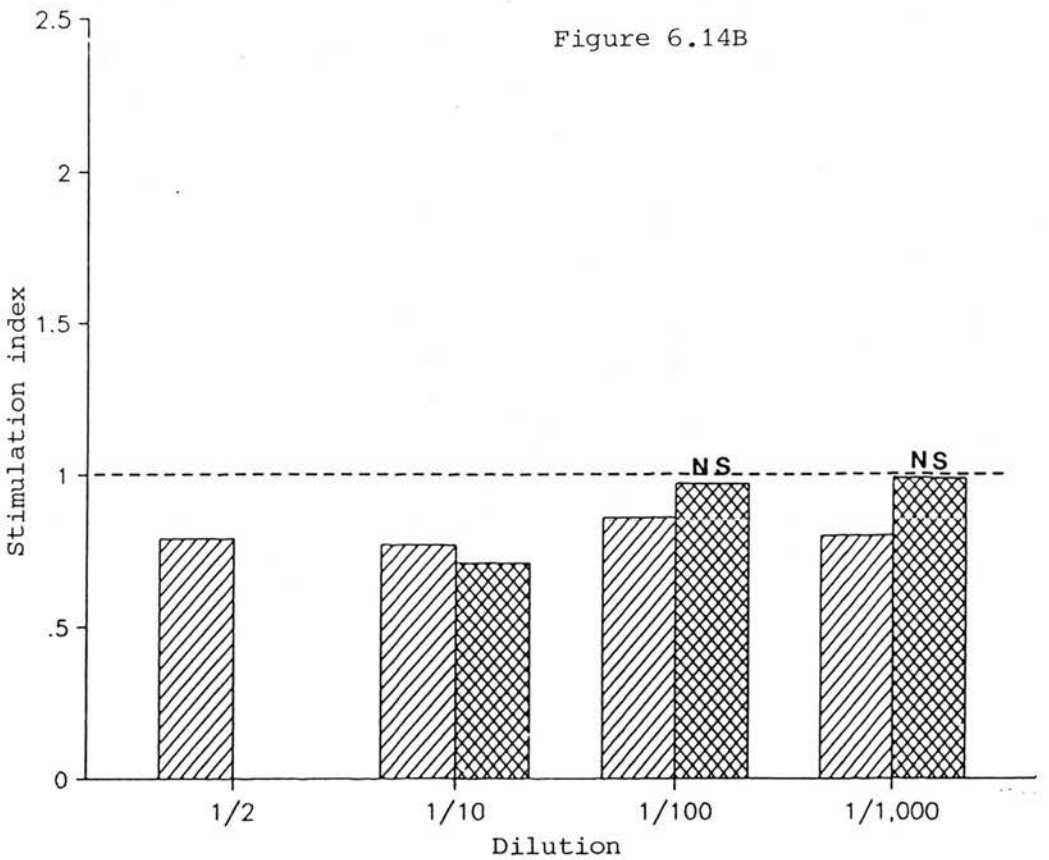


Figure 6.14B



CHAPTER SEVEN

IDENTIFICATION OF LEUCOCYTE PHENOTYPES AT INFECTION SITES

CONTENTS (cont.)

INTRODUCTION

Several studies on the histological characteristics of *D.congolensis*-infected skin have been undertaken and have demonstrated the presence of leucocyte infiltrates in both natural and experimental infections (see chapter 1). To date, no work has been published on the identification of the type of leucocyte present, other than the distinction made between PMN and mononuclear cells and the report of immunoglobulin A-bearing plasma cells in the dermis of experimentally-infected Merino sheep (Ellis *et al* 1987). Until recently, this type of study was not possible because of the non-availability of suitable monoclonal antibodies.

With the identification of the cell types present at an infection site comes an understanding of the immune effector mechanisms likely to be involved in the host response to the disease. A comparison of the cellular infiltrate immunophenotypes in transient and chronic, generalised infections would also allow identification of potential defects associated with the chronic state. The present study involved identification of the leucocyte subsets at the site of an experimentally-induced *D.congolensis* infection, from the time of inoculation through to the resolution of the lesion.

MATERIALS AND METHODS

ANIMALS

Inbred male Wistar rats, approximately three to five months-old were used.

INFECTION OF RATS WITH *DERMATOPHILUS CONGOLENSIS*

D.congolensis was cultured and harvested from 48 hour-growth B/A plates (p.50). Animals were infected three times, at different sites, at ten-day intervals, by the method described previously (p.52). At each time of infection, a control site, on the opposite side of the body to the inoculation site, was subjected to the same procedure of shaving, cleansing with ether and scarification but no *D.congolensis* was applied.

PREPARATION OF SKIN SECTIONS

Samples were taken at daily intervals for seven days and on day ten following initiation of the third infection. The area of skin at the inoculation site and the area at the control site were excised, wrapped in aluminium foil and frozen to -78°C. For comparison, normal skin samples were also taken from a non-infected rat.

Cryostat sections, each 5 µm thick, were cut at -40°C by Mr.Ian Heron. The sections were transferred to glass slides which had previously been coated by dipping into a chrome alum/gelatin solution (Appendix 6) and then allowed to dry overnight at 37°C.

IDENTIFICATION OF CELL TYPES WITH MONOCLONAL ANTIBODIES AND IMMUNOPEROXIDASE LABELLING

The skin sections were allowed to dry for an hour at room temperature or at least 20 minutes at 37°C and were then fixed in 4°C acetone[1]. The sections were rinsed with two ten-minute washes in PBS. Gentle stirring was used for all steps involving washes.

To block endogenous peroxidase activity, the sections were incubated in a 0.1 M sodium azide[1] solution in PBS, containing one per cent hydrogen peroxide[1], for 30 minutes. The sections were washed twice with PBS for ten minutes each and then in buffer containing normal rat serum and BSA (NRS-BSA-buffer, Appendix 6) for 45 minutes.

Duplicate sections were incubated with 10 µl of an anti-rat monoclonal antibody (Table 7.1) for one hour under humidified conditions. The monoclonals had been optimally diluted with NRS-BSA-buffer, stored at 4°C and used within one week of preparation. Prior to use, the monoclonals had been tested at a range of dilutions to determine the optimum.

The sections were washed twice in PBS and then twice in NRS-BSA-buffer; each wash was for ten minutes. Rabbit anti-mouse IgG peroxidase conjugate was then applied, 20 µl per section. The conjugate was an affinity purified $F(ab')_2$ preparation used at the pre-determined optimum concentration of 10 µg ml^{-1} (Appendix 6).

After incubation with the conjugate for one hour under humidified conditions, the sections were washed in NRS-BSA-buffer for 20 minutes, followed by a ten-minute wash in PBS. The substrate, diaminobenzidine solution containing hydrogen peroxide (DAB, Appendix 6) was then applied for ten minutes. Excess substrate was removed by thorough rinsing in purified water and the staining enhanced by incubation with osmium

[1]Analar, BDH.

Table 7.1: Monoclonal antibodies used to identify cell types in rat skin.

Monoclonal	Rat cell determinant recognised by the monoclonal
MRC OX-18	Class I monomorphic MHC antigen (RT-1A)
MRC OX-6	Class II (Ia) monomorphic MHC antigen
MRC OX-19	Glycoprotein on T-lymphocytes
W3/13	Determinant on T-lymphocytes, polymorphonuclear leucocytes and plasma cells
W3/25	Glycoprotein on T-helper cells and some macrophages
MRC OX-8	Glycoprotein on T-cytotoxic/suppressor cells and most natural killer cells
MRC OX-39	Interleukin-2 receptor
MRC OX-33	Leukocyte common antigen exclusive to B-lymphocytes
ED1	Determinant on monocytes, macrophages and dendritic cells
MRC OX-42	Determinant on most macrophages, granulocytes and some dendritic cells
1C7	None
None	None

All monoclonals, except OX-42, W3/13 and 1C7, were mouse IgG1, anti-rat, in the form of ascites (Serotec) and were used at a dilution of 1/100. OX-42 was mouse IgG2a, anti-rat in the form of ascites (Serotec), used at 1/100. W3/13 was a mouse IgG1, anti-rat supernatant preparation, used neat (Seralab). 1C7, a mouse, IgG, irrelevant antibody was also used neat (p.89). MHC represents major histocompatibility complex.

tetroxide (Appendix 6) for two minutes. The sections were washed several times with tap water and counterstained with haematoxylin (Appendix 6) for three seconds, followed by rinsing in tap water. After dehydration in ascending concentrations of ethanol, namely, 70, 90 and twice in 100 per cent, the sections were cleared in Xylene[1] and mounted with DPX[1].

Haematoxylin and eosin stained sections were also prepared from the same skin samples. The sections were processed along with those for immunoperoxidase staining but were not incubated with monoclonals, conjugate, DAB or osmium tetroxide. The sections were rinsed in tap water, incubated in haematoxylin for three minutes, in Scott's tap water substitute for five minutes and in Putt's eosin for ten seconds (Appendix 6); they were then rinsed in tap water, dehydrated in ethanol and mounted.

PREPARATION OF DRAINING-LYMPH NODE SECTIONS

The lymph node which drained the area under the infection site, or for controls, the scarification site, was first identified by the intradermal injection of 0.1 ml of ten per cent Evan's blue dye (Gurr) at the site at which rats were normally infected. Two hours later, the rat was sacrificed and a post-mortem examination carried out. The dye was found concentrated in the lymph node in the axilla.

At the same time that skin samples were taken, the draining-lymph nodes from infected and control sites were removed and processed alongside the skin sections. The only difference was that lymph node sections were cut at -32 to -35°C, rather than at-40°C.

[1] Analar, BDH.

RESULTS

DERMATOPHILUS CONGOLENSIS-INFECTED SKIN

DETERMINATION OF IMMUNOPHENOTYPES WITH MONOCLONAL ANTIBODIES

MRC OX-18

The monoclonal antibody OX-18 recognises the determinant RT-1A, which is a class I antigen of the major histocompatibility complex. Virtually all the structures in the skin were labelled by MRC OX-18 (Figure 7.1). In the dermis the fibroblasts, sebaceous gland cells and the hair follicle cells were positive but the hair shaft itself was negative. The lower layers of the epidermis were labelled, but the outer, more differentiated layers were not; nor was the scab material at the site of infection. The same staining pattern was observed on all days.

MRC OX-6

The monoclonal antibody OX-6 recognises Ia antigen ie. class II. One day after infection, no staining was observed in the epidermis, except for occasional isolated cells but by the second day the epidermis displayed widespread staining (Figure 7.2) except for the outermost layers. By the seventh day after infection, only patchy staining of the epidermal cells was present; this persisted to day ten. Most mononuclear cells in the dermal and epidermal infiltrates, observed from day two onwards, were also labelled by OX-6.

In addition, non-specific staining of dermal fibroblasts and collagen occurred with this monoclonal. Other structures in the skin, such as sebaceous glands, hair follicles and the scab material did not stain.

MRC OX-19

The monoclonal antibody OX-19 is a pan T-cell marker. On the first day after infection, no labelled cells were present. By the second day, small groups of positive cells were visible in the lower dermis. On day three, in addition to the cells in the lower dermis, a few labelled cells were present just underneath the epidermis and occasionally within the epidermis. By day six, OX-19 labelled cells were visible in small groups throughout the dermis as well as scattered below the epidermis along its length and within it. Sometimes the positive cells were grouped in the dermis in a pattern which gave the appearance of a band of cells moving up through the dermis towards the epidermis (Figure 7.3). The OX-19 positive cells persisted throughout the period studied.

No non-specific staining occurred with this monoclonal.

W3/13

The monoclonal antibody W3/13 recognises T-cells, together with polymorphonuclear leucocytes (PMN) and plasma cells. On the first and second days after infection, a mass of W3/13 labelled cells were visible within the epidermis; in some sections most of the epidermis had been lost (Figure 7.4). The same group of cells did not stain with the OX-19 monoclonal suggesting that they were PMN, or plasma cells rather than T-cells. Some labelled cells were also visible just underneath the epidermis. W3/13 also recognised most of the cells labelled by OX-19.

No non-specific staining occurred with this monoclonal.

W3/25

The monoclonal antibody W3/25 labels T-helper cells and some macrophages. In infected skin the pattern of labelling was broadly

similar to that of OX-19. However some of the cells labelled by W3/25 were not recognised by OX-19, such as those under and in the epidermis on day two of infection. From day two to day ten inclusive, W3/25 positive cells were present within and just under the epidermis and throughout the dermis (Figures 7.5 and 7.6). Small groups of labelled cells were particularly abundant in the dermis on days six and seven.

Unfortunately, W3/25 also gave non-specific staining of dermal fibroblasts and collagen, other skin structures were negative. Nevertheless, on the basis of shape, one could easily differentiate between labelled mononuclear cells and the non-specific staining.

MRC OX-8

The monoclonal antibody OX-8 recognises T-cytotoxic/suppressor cells. OX-8 positive cells were first observed in the dermis on day two of infection and persisted until day ten. Unlike the W3/25 cells, none of the OX-8 positive cells were observed just underneath the epidermis until day six. Furthermore, positive cells were seldom observed within the epidermis (Figure 7.7).

OX-8 gave a similar pattern of non-specific staining as W3/25.

MRC OX-39

The monoclonal antibody OX-39 is specific for the interleukin-2 receptor, present on most activated T-cells. OX-39 positive cells were apparent on day two of infection and followed the same pattern of labelling as the W3/25 and OX-8 cells (Figure 7.8), up until day ten, when no cells were labelled apart from some weakly stained cells in the lower dermis.

Some non-specific staining of dermal fibroblasts and collagen occurred

with this monoclonal. As with all the other antibodies, it was easy to differentiate between labelled mononuclear cells and non-specific staining.

MRC OX-33

The monoclonal antibody OX-33 recognises B-cells. No OX-33 infiltrate occurred within the ten days studied. No non-specific staining occurred with this monoclonal. The lack of labelling was not due to non-functioning of OX-33 (see p.200).

ED1

The monoclonal antibody ED1 will label monocytes, macrophages and dendritic cells. ED1-positive cells were first observed, under and in the epidermis and throughout the rest of the dermis on day two of infection (Figure 7.9). Thereafter, labelled cells were only occasionally observed in the epidermis. The pattern of staining did not always correlate with that of W3/25; for example, some W3/25-positive cells were observed in the epidermis on days six and seven but these did not stain with ED1. In addition, many more cells were stained by W3/25 than by ED1. By day seven few ED1-labelled cells remained.

Some non-specific staining of dermal fibroblasts and collagen occurred with this monoclonal.

MRC OX-42

The monoclonal antibody OX-42 recognises granulocytes, most macrophages and some dendritic cells. The same groups of cells in the epidermis were labelled with OX-42 and with W3/13 on days one and two, suggesting that these were PMN (Figure 7.10). This was confirmed by the non-labelling of

the cells in the epidermis on day one by the other anti-macrophage monoclonal ED1. OX-42 positive cells were observed throughout the dermis from day two onwards although by day ten the staining had become weak. OX-42 also gave some non-specific staining of dermal fibroblasts and collagen.

CONTROL ANTIBODIES

No peroxidase staining was visible in any of the sections, whether from infected, control or normal skin, or from lymph nodes which had been incubated with the irrelevant monoclonal, 1C7 or which had just received the conjugated second antibody (Figure 7.11).

NORMAL AND CONTROL SKIN

The skin samples from non-infected rats and those from control, scarified areas displayed the same pattern of staining with the anti-class I monoclonal, OX-18, as that for the infected skin.

In the presence of anti-class II monoclonal, OX-6, the epidermis in both normal skin and non-infected scarified skin was negative, apart from occasional, isolated positive cells which may have been Langerhans cells.

With the other monoclonals positive cells were rarely observed in normal or control skin. Occassionally a single positive cell was visible in the lower dermis of an entire section.

In the presence of anti-interleukin-2 receptor monoclonal, OX-39, no positive cells were observed in any of the skin sections from normal or control skin.

Those monoclonals which gave some non-specific staining of the dermal fibroblasts and collagen, namely OX-6, W3/25, OX-8, OX-39, ED1 and OX-42, also gave non-specific staining in the normal and control skin. However,

except for OX-6, the staining was very slight compared with that in infected skin.

DRAINING LYMPH NODE SECTIONS

The lymph node which drained the area of *D.congolensis* infection was much enlarged compared with that draining the scarified, control area up until day five of infection. No obvious differences in labelling patterns with the various monoclonals occurred between the nodes from infected and control areas, except for a greater density of cells in the former.

Lymph nodes labelled with OX-33, the B-cell marker, displayed localized staining of the outer cortex and of follicles (Figure 7.12). Whereas, the T-cell markers, OX-19, W3/13, W3/25 and OX-8, recognised cells in the paracortex. OX-39, the interleukin-2 receptor, labelled some cells in the paracortex and none in the follicles or outer cortex. OX-18 and OX-6 both stained the majority of leukocytes. ED1 and OX-42 positive cells were scattered throughout the lymph nodes, but mostly in the cortical area.

Those mononclonals which gave non-specific staining of fibroblasts and collagen in the infected skin, did not produce any non-specific staining in the lymph nodes.

HAEMATOXYLIN AND EOSIN-STAINED SECTIONS

Following application of *D.congolensis* to scarified, cleansed skin, notable differences from normal or scarified, control skin were observed over the period studied. On day one, the epidermis was oedematous with many of the cells appearing necrotic and swollen. Small areas of cellular infiltrate composed of PMN, were enclosed within the epidermis (Figure 7.13). Filaments of *D.congolensis* were visible in the *stratum corneum*.

By day two of infection, the oedema and necrosis had progressed (Figure

7.14). *D.congolensis* filaments were visible both in the *stratum corneum* and the scab which had formed (Figure 7.15). On days three and four, the entire length of the epidermis appeared necrotic and a mononuclear cell infiltrate had collected in the dermis. The scab above the epidermis contained both *D.congolensis* filaments and PMN. By the fifth day, much of the scab had detached and that which remained was composed of about seven layers of *stratum corneum* and some PMN, but no visible filaments. On days six and seven, areas of the epidermis seemed to be detaching from the rest of the skin and a new epidermis was spreading out from the cells of the hair follicles. By this stage, the dermal infiltrate was pronounced. By the tenth day some necrotic epidermis was still present and areas of intact epidermis were acanthotic although this was partly due to oedema. A sample taken on day 18 displayed an intact but thickened epidermis.

The epidermis of the non-infected skin appeared healthy and intact throughout apart from minor disruptive changes observed on day one. In many of the sections, whether from normal, infected or non-infected skin, the epidermis had lost most of the cornified layers during processing (Figure 7.16).

DISCUSSION

Dermatophilus congolensis-infection of skin is followed by an inflammatory reaction, characterised by the localised presence of large numbers of PMN, recognised by the monoclonals W3/13 and OX-42. For as long as the scab remains attached, PMN can be identified within it, although they are not necessarily active throughout.

From the second day onwards after infection, the lesion was characterised by a mononuclear cell infiltrate composed of T-lymphocytes and macrophages. Both T-helper and T-cytotoxic/suppressor cells were present in the infiltrate in large numbers, with the T-helper cells predominating higher up in the skin. Absolute numbers were not determined, so it is not possible to predict whether the overall effect was likely to be upregulation or suppression of the immune response.

The correlation between labelling of interleukin-2 receptor and T-helper and T-cytotoxic/suppressor cells suggests that both types of T-cell were active, since OX-39 will bind to active but not resting T-cells (Osawa and Diamantstein 1983). *D.congolensis-infection* shares some similarities with dermatophyte infections and if the immune effector mechanisms were the same, this would suggest that activated T-helper cells may have been responsible for the resolution of the lesion. Antigen-specific T-helper cells conferred a degree of protection against *Trichophyton quinckeanum* infection, although the actual mechanism of action was not established (Calderon and Hay 1984).

The state of T-cell activation, revealed by the expression of OX-39, suggests a variety of lymphokines were being secreted at the site. Pearsall, Sundsmo and Weiser (1973) demonstrated that supernatants from mitogen-stimulated lymphoctye cultures were cytotoxic to *Candida*

albicans. In contrast, supernatants from mitogen or trichophytin-stimulated lymphocyte cultures were not inhibitory to the growth of *Trichophyton mentagrophytes*, although cytotoxicity towards fibroblasts was demonstrated (Artis and Jones 1980). Jones and Artis (1981) suggested that lymphokine-induced damage of epidermal cells might cause the release of unsaturated transferrin and α_2-macroglobulin, both of which are fungal-growth inhibitors. Such a mechanism may conceivably inhibit the growth of *D. congolensis*.

It would seem unlikely that the activated T-cytotoxic/suppressor cells which were observed could have been directly cytotoxic to *D. congolensis* because of the spatial separation of the two with the pathogen apparently confined to the *stratum corneum*. T-suppressor cells would be expected to predominate only late-on in the host response to infection.

Although the mononuclear infiltrate persisted to the end of the study, the disappearance of IL-2 receptor labelling by day ten may suggest that the state of activation had ceased. This could be a result either of the disappearance of active cells and replacement by non-active cells, because receptors were no longer expressed, or because IL-2 produced by activated T-cells at the infection site, has bound to and therefore blocked all the IL-2 receptors. The latter, however, does not preclude the possibility that the cells were still active. Furthermore, some mononuclear cell class II expression persisted to day ten suggesting that some cells were still active.

The role of the macrophages, identified by ED1 and OX-42 is also unknown. *D. congolensis* has been observed within macrophages but only in atypical, subcutaneous lesions in a human (Albrecht et al 1974) and in a sheep subcutaneously inoculated with *D. congolensis* (Momotani, Inui, Ishikawa and Azuma 1984). It seems unlikely that macrophages would play

an important role in phagocytic removal of *D.congolensis* in normal infections because of the usual distance which separates the two. A more likely role would be augmentation of the immune response by the release of mediators such as interleukin-1.

The labelling of the epidermis by OX-6 in infected skin correlates with other diseases; class II expression by epidermal cells has been noted in a wide range of human skin disorders where a lymphocytic infiltrate is present (Aubock, Romani, Grubauer and Fritsch 1986). Of the ten infectious diseases they examined, class II expression occurred in all of them. The appearance of class II may be caused by IFNγ secreted by activated T-cells (Shimada and Katz 1988), which will induce epidermal class II expression *in vitro* (Basham, Nickoloff, Merigan and Morhenn 1984). The functional significance of epidermal class II is debatable, unlike other class II-positive cells such as Langerhans cells, antigen-processing capacity appears to be minimal (Gaspari and Katz 1988). However, class II-expressing epidermal cells may function to attract Langerhans cell precursors (Daynes, Spangrude, Roberts and Krueger 1985) and possibly lymphocytes into the skin (Roberts, Daynes and Krueger 1984). The labelling of mononuclear cells by OX-6 was expected since macrophages and 40-60 per cent of activated T-cells express class II (Stingl, Katz, Shevach, Rosenthal and Green 1978 and Charron, Engleman, Benike and McDevitt 1980).

The lack of a B-lymphocyte infiltrate may simply have been a reflection of the time period studied, in that B-cells might have occurred in the skin later in the reaction. Alternatively, the infiltration may follow the pattern of other skin disorders such as atopic dermatitis (Lever, Turbitt, Sanderson and MacKie 1987) and psoriasis (Stingl, Wolff, Diem, Baumgartner and Knapp 1977) where B-cells are seldom observed. Likewise,

delayed hypersensitivity reactions to tuberculin are characterised by an infiltrate of T-cells but an absence of B-cells (Poulter, Seymour, Duke, Janossy and Panayi (1982). Indeed, B-cells do not have the homing capacity shown by T-cells which attracts the latter to inflammatory skin sites (Van Dinther-Janssen, Van Maarsseveen, De Groot and Scheper 1983). The lack of OX-33 labelling was not due to a failure of the monoclonal to recognise B-cells. This was demonstrated by the specific staining of lymph node follicles and outer cortex, the sites at which B-cells localise (Barclay 1982).

Normal rat skin contained extremely few mononuclear or polymorphonuclear cells, often less than one per entire section. Likewise leucocytes were rarely observed in non-infected skin which had undergone the same preparative procedures as the infected sites. This was surprising, since an early inflammatory reaction to the local tissue damage was to be expected. The lack of a noticeable reaction may have been a result of the sections missing the area of damage, or of the small amount of trauma caused by the light scarification.

The non-specific staining of fibroblasts and collagen by some monoclonals proved impossible to eliminate although it was reduced when the concentration of sodium azide in the hydrogen peroxide solution was increased to 0.1 M, as recommended by Malorny, Bildau and Sorg (1988). There are several possible reasons for the non-specific staining but because it was very noticeable only in the samples from infection sites, the most likely is endogenous peroxidase activity. Both granulocytes and macrophages contain peroxidase, are often abundant at inflammatory sites and will give rise to non-specific staining (*Ibid.*). However, this does not explain why non-specific binding occurred with only some of the monoclonals, whereas there was none whatsoever with others or with the

control antibodies. The labelling of the fibroblasts by some monoclonals may represent true cross-reactivity since some fibroblasts in non-infected skin were stained, with the rest of the section completely negative.

Dermatophilus congolensis filaments could be observed in the *stratum corneum* and scab in the haematoxylin and eosin-stained sections on days one to four. One cannot rule out the possibility that the organism remained after this time or that it invaded deeper than the *stratum corneum* because *D.congolensis* is difficult to distinguish in tissue sections when non-bacteriological stains are used, particularly if the filaments have dispersed into cocci. The fact that filaments were visible over four days provides evidence that *D.congolensis* was actively growing, since it was originally applied as a suspension of cocci.

This study centred on animals which had previously been exposed to *D.congolensis*. An earlier, histological study of primary infection sites revealed the same early reaction of a PMN infiltrate followed by a mononuclear cell response, but the latter was comparatively late and was not noticeable until day five of infection (Morrow *Pers Comm*).

Polymorphonuclear cells, followed by activated T-helper and T-cytotoxic/suppressor cells, together with macrophages infiltrate the skin at the site of *D.congolensis*-infection. The role of these different cell types in the host response is, at present, a matter for speculation.

FIGURE 7.1 Labelling pattern of OX-18, which recognises class I determinants, on rat skin.

Immunoperoxidase-stained cryostat section, taken from a rat on day ten of infection, showing:

E	Epidermis
D	Dermis
S	Sebaceous gland
HF	Hair follicle
HS	Hair shaft

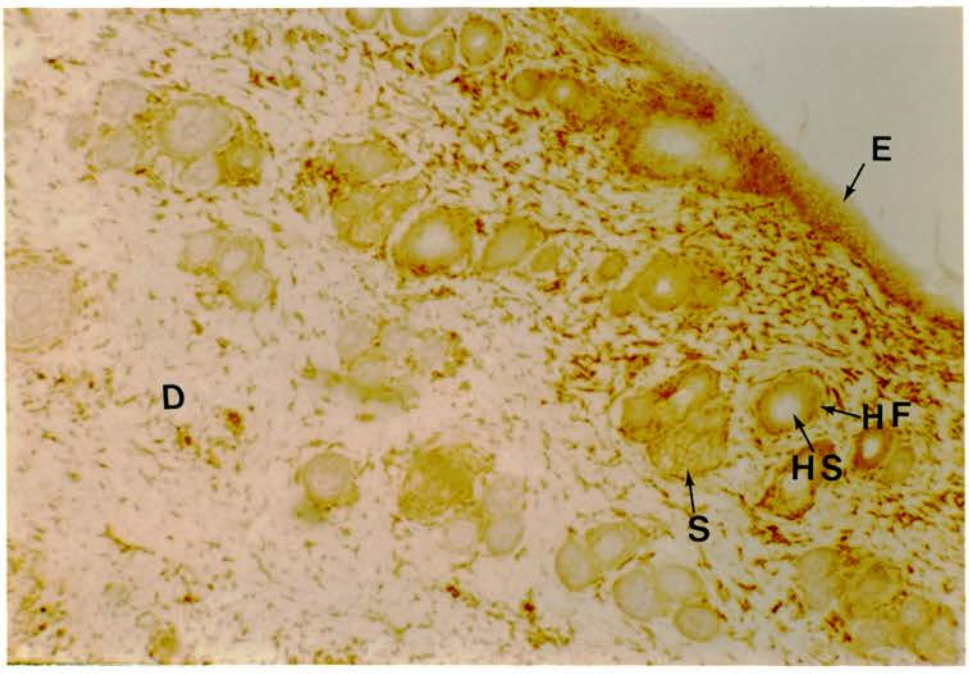
(x 65 magnification)

FIGURE 7.2 Class II expression by rat epidermis at the site of *D.congolensis* infection. The section was taken on day six of infection.

OX-6 labelled epidermal cells (closed arrows)
OX-6labelled mononuclear cells in the dermis (open arrows)

Immunoperoxidase-stained cryostat section

(x 425 magnification)


E
D
HF
HS
S

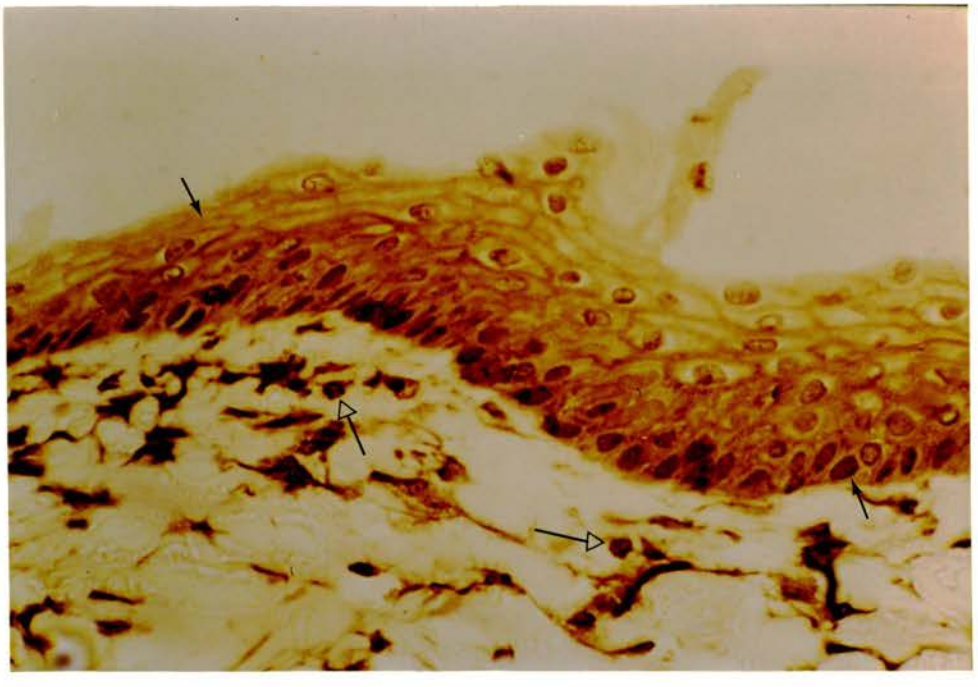


FIGURE 7.3 Groups of OX-19 labelled mononuclear cells (arrows) in the dermis of a rat on day seven of infection.
OX-19 is a pan-T cell marker

Immunoperoxidase-stained cryostat section

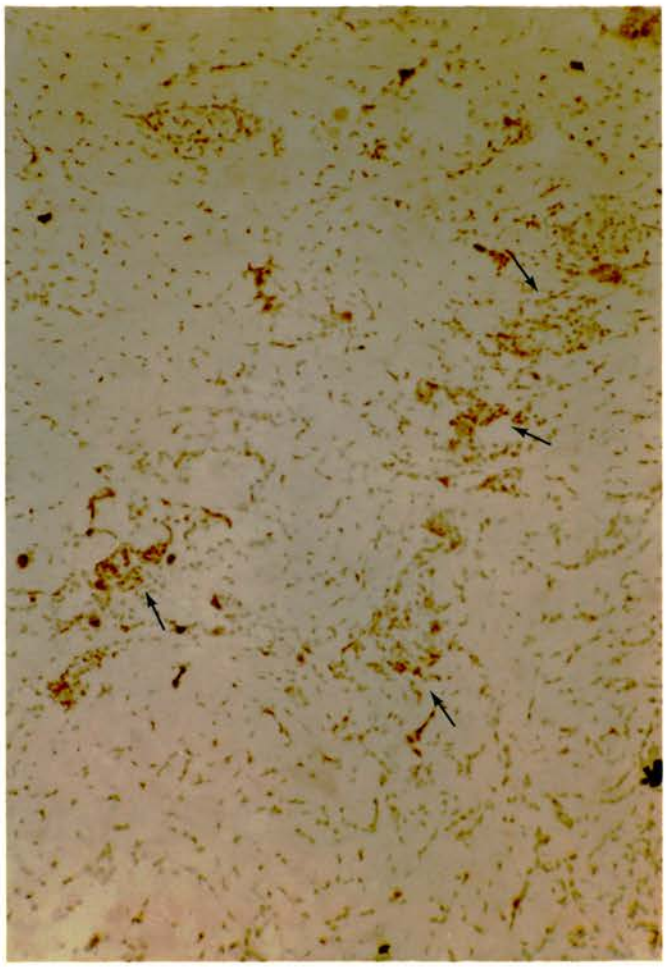
(x 106 magnification)

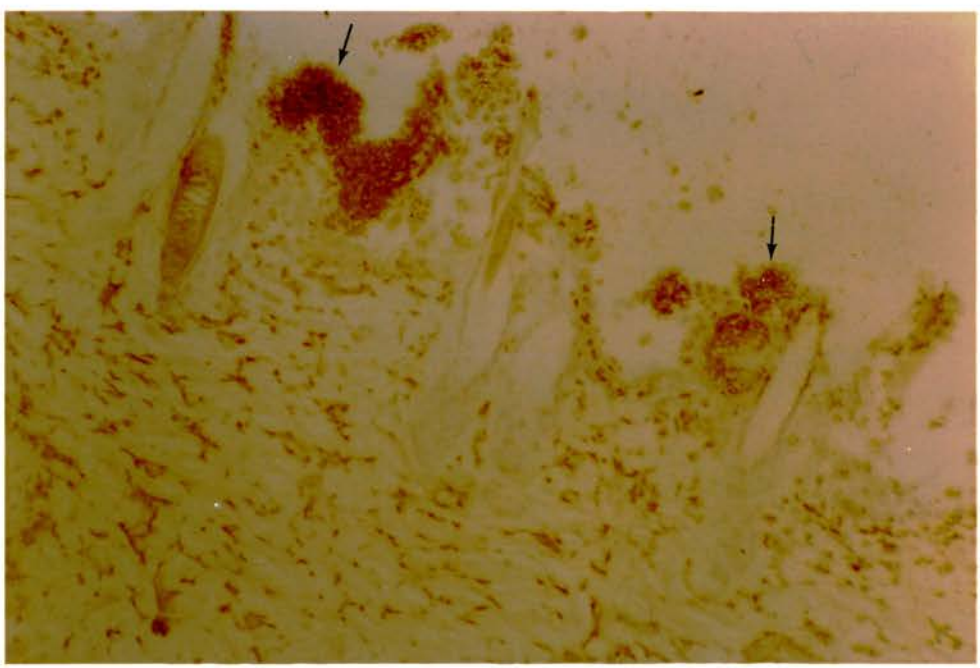
FIGURE 7.4 Mass of W3/13-labelled cells (arrows) immediately below the site of the epidermis which has been lost. The section was taken from a rat on day two of infection. The cells co-stained with OX-42 and are therefore probably polymorphonuclear leucocytes (PMN). W3/13 recognises PMN and T-cells.

Immunoperoxidase-stained cryostat section

(x 106 magnification)

The sections in figures 7.3 and 7.4 were over-stained with the counter stain, haematoxylin.



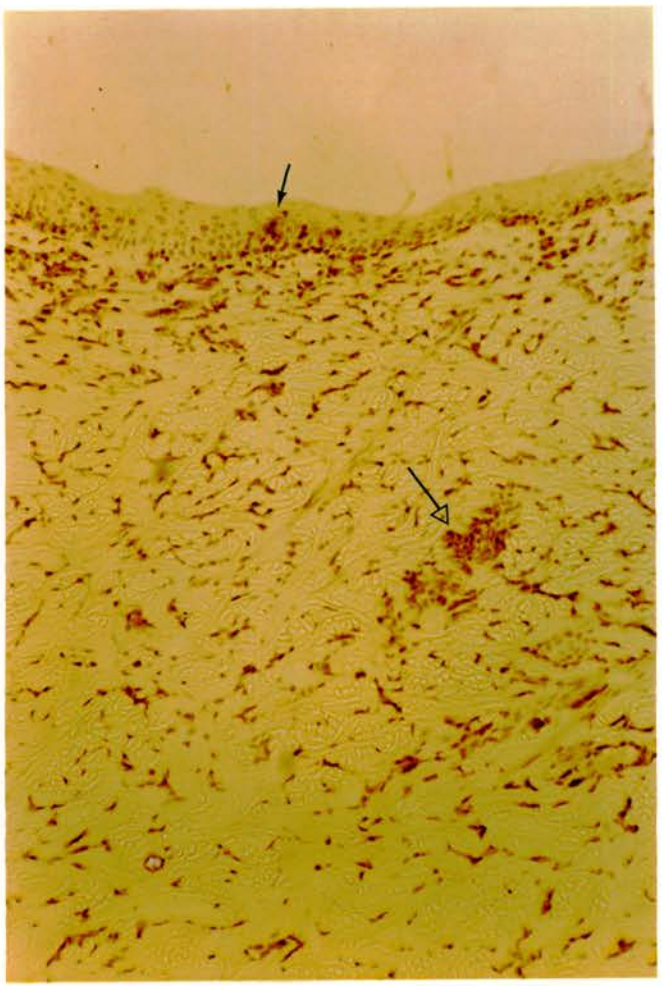


FIGURES 7.5 and 7.6 W3/25-positive cells in the dermis (open arrows) and epidermis (closed arrows) of a rat on day six of infection.
W3/25 recognises T-helper cells and some macrophages.

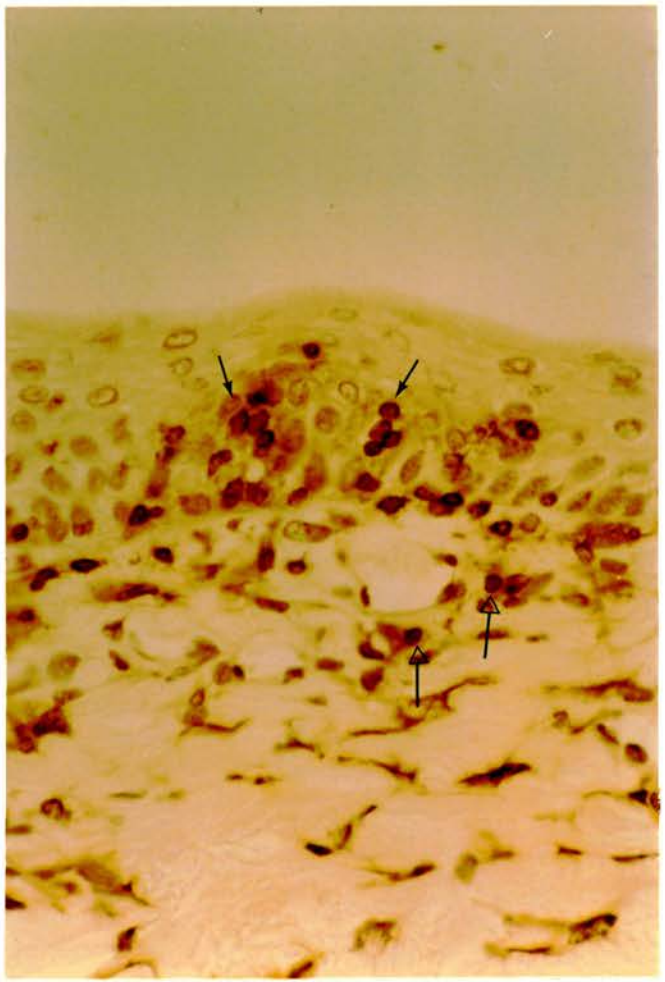
Immunoperoxidase-stained cryostat section

Figure 7.5 x 106 magnification

Figure 7.6 x 425 magnification



7.5



7.6

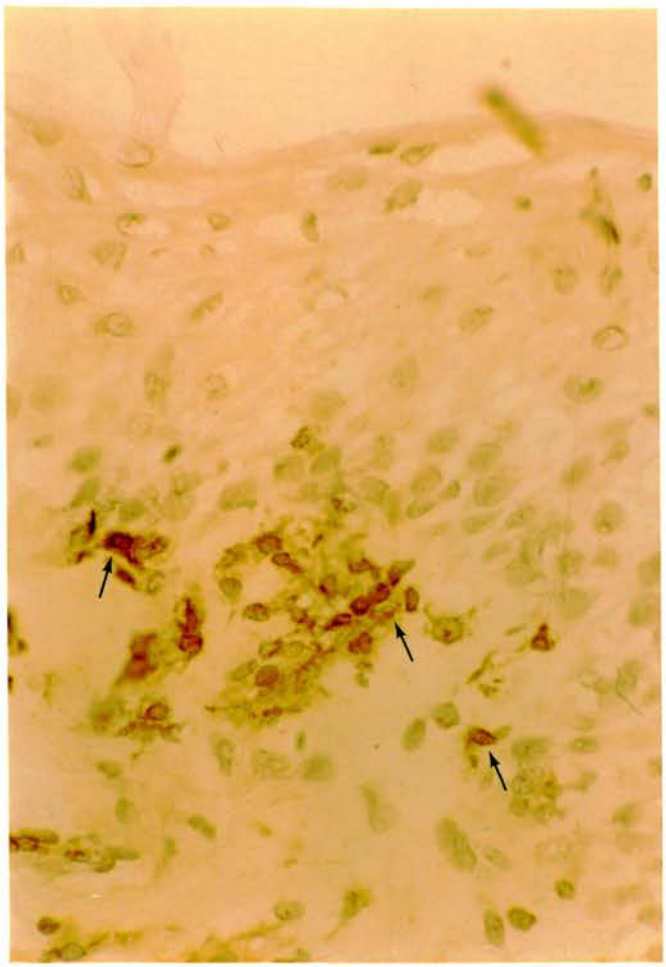
FIGURE 7.7 OX-8-labelled cells (arrows) just underneath the epidermis of a rat on day six of infection.
OX-8 recognises T-cytotoxic/suppressor cells and most natural killer cells.

Immunoperoxidase-stained cryostat section
(x 425 magnification)

FIGURE 7.8 Interleukin-2 expressing (OX-39 labelled) cells (arrows) in the dermis of a rat on day seven of infection.

Immunoperoxidase-stained cryostat section

(x 425 magnification)



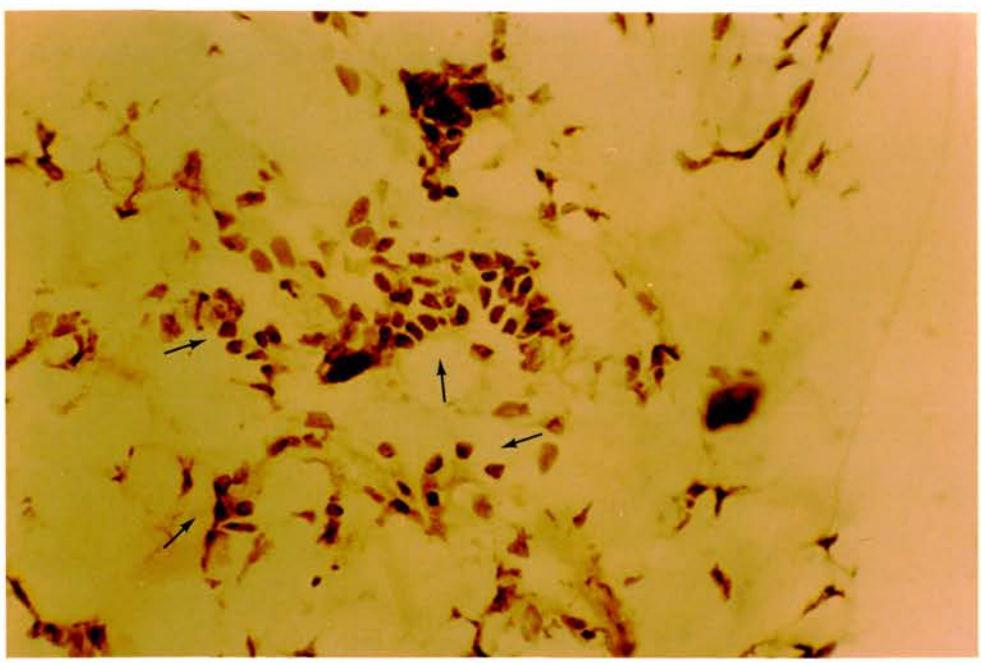


FIGURE 7.9 Day two infection, ED1-labelled cells (closed arrows) situated in the epidermis underneath the scab (open arrow);
ED1 recognises macrophages, monocytes and dendritic cells.

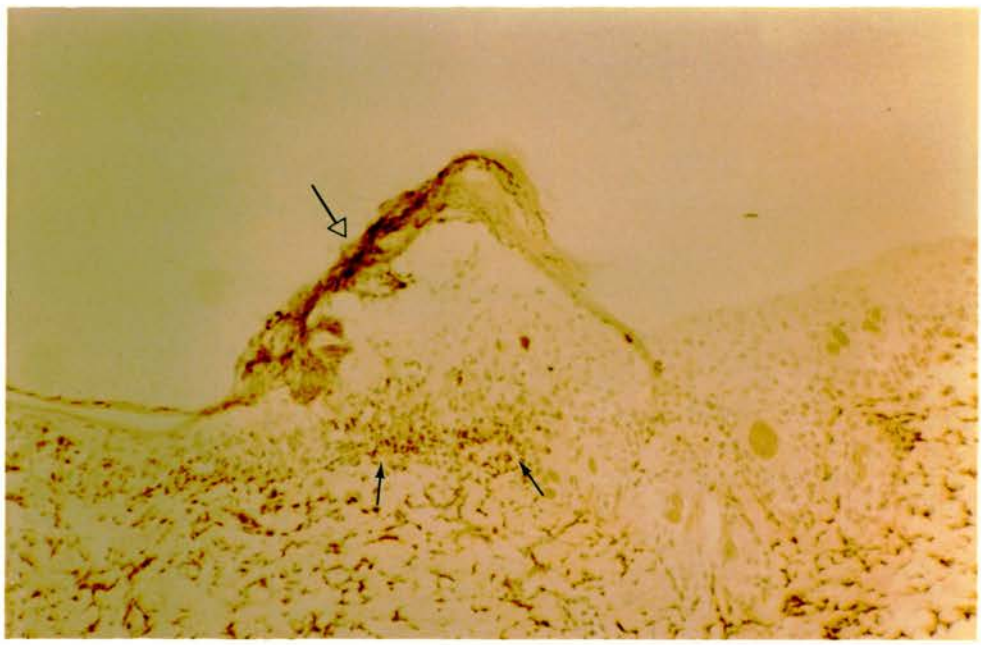
Immunoperoxidase-stained cryostat section

(x 106 magnification)

FIGURE 7.10 OX-42-labelled cells in the epidermis and scab of a rat on day two of infection.
OX-42 recognises granulocytes and most macrophages and dendritic cells.

Immunoperoxidase-stained cryostat section

(x 106 magnification)



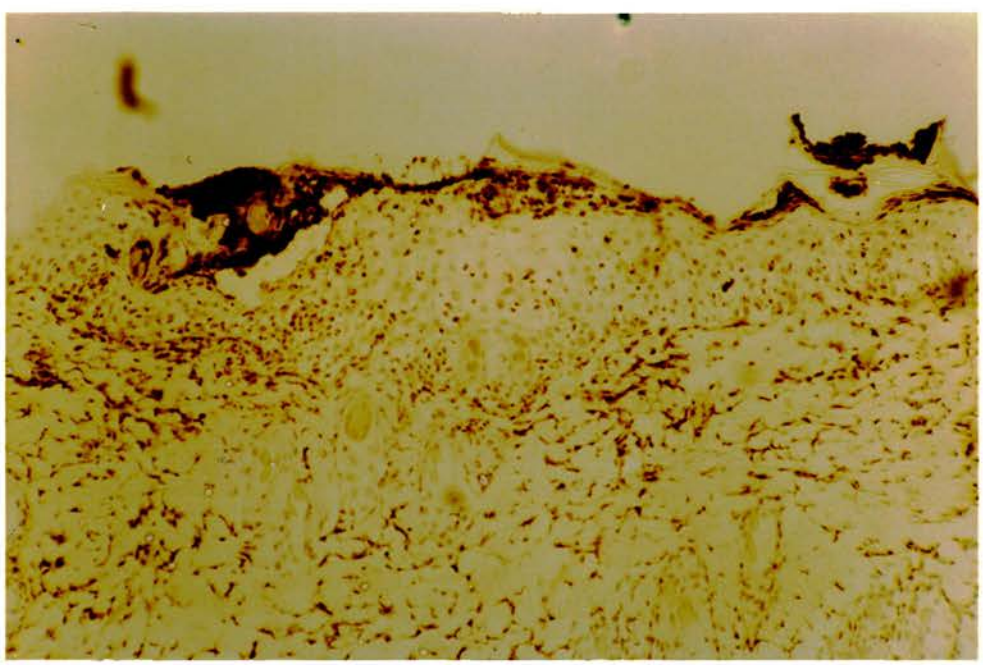


FIGURE 7.11 Rat skin on day three of infection incubated with the negative control antibody, 1C7. No structures are stained.

Immunoperoxidase-stained cryostat section.
(x 106 magnification)

FIGURE 7.12 OX-33 labelling of the lymph node draining the site of infection taken on day six. The labelling is confined to the follicles (arrows) and outer cortex which are the sites of B-cell localization.

Immunoperoxidase-stained cryostat section

(x 65 magnification)

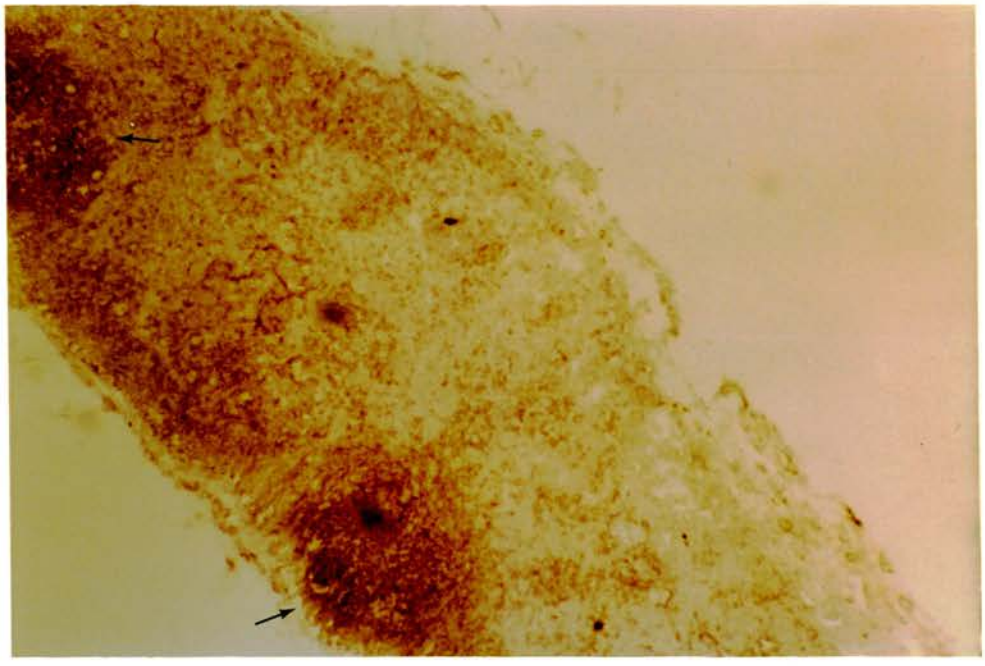


FIGURE 7.13 Haematoxylin and eosin-stained cryostat section of rat skin on day one of infection.
Pockets of PMN were present within the epidermis (arrows).

(x 65 magnification)

FIGURE 7.14 Haematoxylin and eosin-stained cryostat section of rat skin on day two of infection, showing oedema and necrosis of the epidermis (closed arrows) and the presence of a scab (open arrow).

(x 65 magnification)

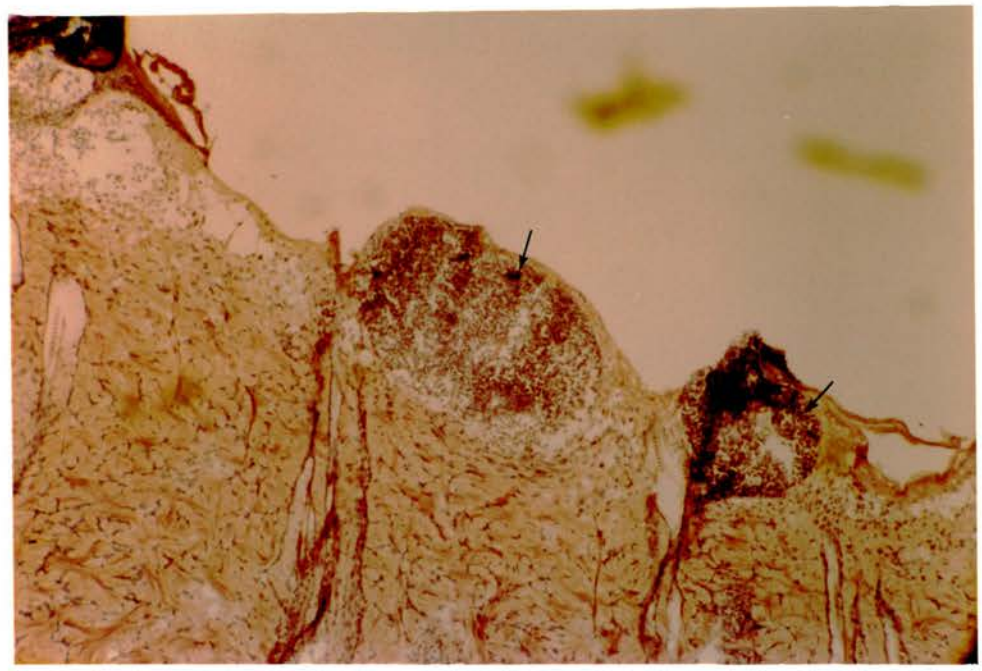


FIGURE 7.15 Haematoxylin and eosin-stained cryostat section of rat skin on day two of infection, showing *D.congolensis* filaments within the *stratum corneum* (arrows).

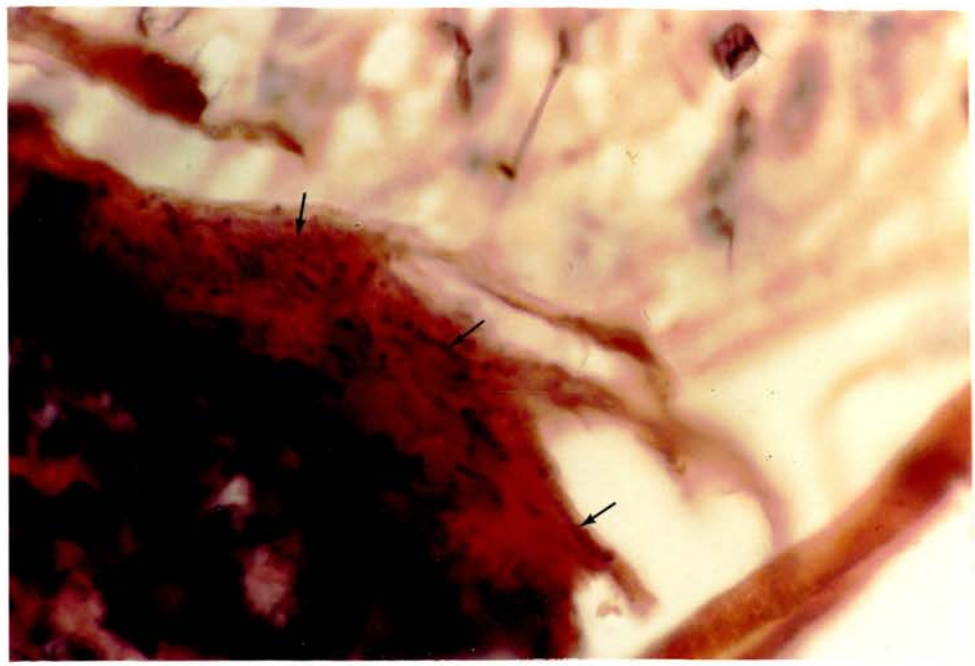
(x 1,060 magnification)

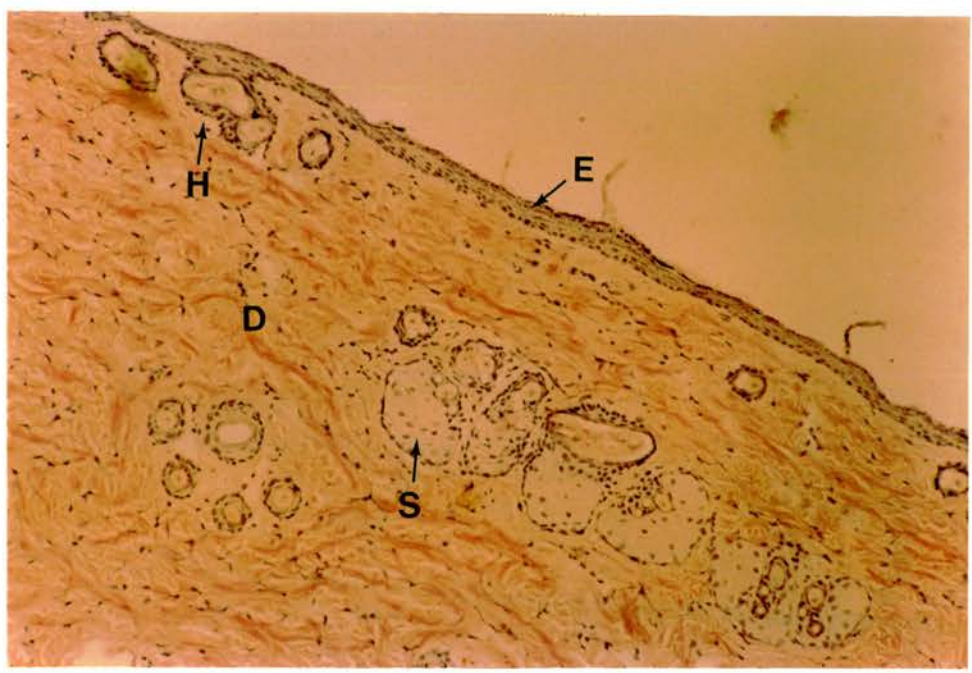
FIGURE 7.16 Haematoxylin and eosin-stained cryostat section of normal rat skin.

E Epidermis
D Dermis
S Sebaceous gland
H Hair follicle

The outermost layers of the epidermis have been lost during processing.

(x 65 magnification)




H
E
D
S

GENERAL DISCUSSION AND SUMMARY

Dermatophilosis encompasses a wide spectrum of symptoms in terms of severity and of duration in which a range of intercurrent factors, both external and host-related appear to modulate the outcome of infection. The present study has concentrated on one part of the disease spectrum, namely, that of the transient, localised infection, experimentally induced through a break in the integrity of the skin barrier. The rationale for the study was the belief that the host reaction responsible for resolving the experimentally-induced infection is likely to be of the same type as that found in a successful response to a natural infection. An understanding of the host response that results in rapid clearance of *D.congolensis* logically precedes an investigation of the defects in host defence mechanisms that allow establishment of chronic, generalised dermatophilosis.

Mononuclear cells, from animals with an experimentally-induced acute infection, underwent a strong and specific *in vitro* proliferative response to *D.congolensis* (Chapter 2). Thus, it would appear that *D.congolensis* is not itself immunosuppressive and, under normal conditions, an immune response is quickly elicited. In contrast, mononuclear cells from cases of generalised, chronic dermatophilosis may well not respond to *in vitro* stimulation. This view is supported by the absence of an *in vitro* lymphocyte response to specific antigen observed in chronic dermatophyte infections (Hanifin *et al* 1974) and in some types of patient with chronic mucocutaneous candidiasis (Kirkpatrick and Sohnle 1981). However, depressed lymphocyte transformation to specific antigen does not always correlate with the chronic state of skin diseases normally combated by cell-mediated immunity. The *in vitro* lymphocyte

response to dermatophyte antigen, in patients suffering from a chronic infection, has been noted to vary according to the dermatophyte species and the site of infection (Hay and Shennan 1982).

The exact relationship between the immune response to experimentally induced infection and the rapid resolution of the lesion is still unclear. Certainly, PMN, T-helper, T-cytoxic/suppressor and macrophages are attracted to the site of infection and the T-cells, at least, are in a state of activation (Chapter 7). *D.congolensis* may be combated by a variety of phagocytic, cytotoxic and growth-inhibitory mechanisms mediated by these cells but further investigation is required to elucidate the relative importance of each and the interactions between the different cell types in the host response. A series of cell-transfer experiments would allow identification of the cell types responsible for resolving an infection. Firstly, reliable indices of the degree of infection must be defined; one, for example, could be the number of motile *D.congolensis* cocci recoverable from the infection site, as suggested by Davis (1984). Others could be the duration of infection, the area of the lesion and the minimum infective dose. Secondly, lymphoid cells taken from donor animals which had undergone experimentally-induced infection, could be transferred to syngeneic recipients, previously depleted of lymphoid cells by sub-lethal irradiation. If the cell transfer resulted in enhanced recovery compared with controls, ie. if immunity could be adoptively transferred, the next step would be to identify the active cell type. Lymphoid cells, taken from donors as above, would be treated with one of the anti-leucocyte monoclonal antibodies and complement, to deplete the transferred cells of the subset recognised by the monoclonal. The index of infection severity could then be compared with that for each of the other monoclonal-depleted

recipients and with the controls.

The type of experiment described above was used by Calderon and Hay (1984) who demonstrated that T-cells, which were non-cytotoxic/suppressor, were able to confer a degree of protection against infection by the dermatophyte *Trichophyton quinckeanum*. *Dermatophilus congolensis* infection shares several features with dermatophyte infection. In neither does complete immunity to the pathogen develop but re-infection by *D.congolensis* is often followed by accelerated healing which is associated with the onset of delayed-type hypersensitivity (Roberts 1966a). Likewise, cell-mediated immunity is responsible for the accelerated healing which occurs on re-exposure to dermatophytes (Jones and Artis 1981). Other aspects of *Dermatophilus* infection such as the occurrence of both transient and chronic syndromes, the localization of the organism in the upper layers of the epidermis (Amakiri 1974) and the development of a non-protective humoral response (Roberts 1966a) are also shared by dermatophyte infections although antibody responses are not always present in the latter (Reviewed by Jones and Artis 1981).

Infection with *D.congolensis* is followed by tissue damage, in particular to the epidermis (Chapter 7); this is attributable to a combination of self-trauma due to the inflammatory reaction and direct injury caused by the pathogen, although it is not certain that *D.congolensis* is keratinolytic. The extent of damage is such that areas of the epidermis are replaced by outgrowths from the hair follicle sheath (Oduye 1976b). In experimental infections the new epidermis does not usually become invaded and *D.congolensis* is shed from the skin along with the old epidermis. Several factors may be responsible for the apparent inability of *D.congolensis* to invade the newly-formed epidermis in experimentally-induced infections. One factor may be a response by the

epidermis itself occurring in addition to, or perhaps even induced by, the presence of the cellular infiltrate. An increased proliferation rate, with subsequent enhanced desquamation, possibly also with increased keratinisation may hinder the establisment of *D.congolensis*.

The potential involvement of the host immune response to *D.congolensis* with epidermal defence mechanisms was investigated following the development of a culture system comprised of epidermal cells susceptible to growth-regulatory factors (Chapter 5). Mononuclear cells derived from experimentally-infected rats stimulated *in vitro* with *D.congolensis*, produced both growth-enhancing and growth-inhibitory factors for epidermal cells, the predominant effect being concentration-dependent (Chapter 6). Some of these factors would probably be produced at the site of infection; if so, the net effect of growth-stimulation or inhibition would be a function of the size of the infiltrate, the degree of activation and the distance of the activated cells from the proliferative cells of the epidermis.

Although it is impossible to relate the *in vitro* concentration of growth-influencing factors with that *in vivo*, one may assume that, if the secretory cells were nearby, a relatively high concentration of factors would result in the vicinity of the epidermal proliferative cells. The overriding influence of the supernatants, when at the highest concentration, was stimulation of epidermal growth at normal calcium levels. The mononuclear cell response to *D.congolensis* was largely due to proliferation of T-helper cells (Chapter 3) and these were actively secreting lymphokines (Chapter 4). Thus, the mononuclear cells responsible for the release of the growth-influencing factors were presumably mostly T-helpers and during the course of infection these were found immediately underneath and within the epidermis (Chapter 7).

A similar model was described by Valdimarsson, Baker, Jónsdóttir and Fry (1986) for the hyperproliferative skin disease, psoriasis. They proposed that the increased epidermal proliferation was caused by the influx of activated T-helper cells into the epidermis. The proliferation of the epidermal cells would result in an increase of IL-1 synthesis by these cells, which in turn would induce further proliferation (*Ibid.*). Ristow (1987) was also of the view that epidermal hyperproliferation was a result of stimulation by lymphokines. He proposed that IL-1, released from monocytes in the course of an inflammatory reaction, directly caused increased proliferation and subsequent epidermal thickening. Other lymphokines which might affect epidermal growth were discussed in chapter six.

In contrast, Morhenn (1988) proposed that lymphokines may exert an indirect effect on epidermal growth. At an inflammatory site, fibroblasts might secrete large amounts of somatomedin-C which would increase epidermal proliferation. The basis for the model was that IL-1 stimulates fibroblast proliferation (Schmidt *et al* 1982) and increased numbers of fibroblasts would then secrete more somatomedin-C, a substance which stimulates epidermal growth (Nickoloff, Misra and Morhenn, cited by Morhenn 1988). The demonstration of growth-factors released by specifically-activated mononuclear cells (Chapter 6) supports a direct role for the immune response in the regulation of epidermal proliferation during skin infections but this does not preclude the possibility that immune-mediated and non-specific inflammatory reactions may act in concert. Nevertheless, the contribution of the immune response to epidermal defence is highlighted by the demonstration of epidermal hyperproliferation in response to cutaneous *Candida albicans* infection which is significantly greater in immune than in naive animals (Sohnle

and Kirkpatrick 1978). Furthermore, *Trichophyton mentagrophytes* infection induces epidermal hyperproliferation in guinea-pigs, the onset of which is accelerated on re-infection and, in both primary and secondary infections, correlates with the time of healing (Tagami 1985).

In view of the specific response of T-helper cells to *D.congolensis* demonstrated in this study of a transient infection (Chapters 2,3 and 4) two types of host reaction can be proposed resulting in either acute or chronic syndromes. Thus, when the end result is a transient infection, the initial exposure is followed by antigen-processing and presentation and the generation of antigen-specific T-cells. On a second exposure to the organism, antigen-specific, as well as naive, lymphoid cells would be recruited to the site and mechanisms such as enhanced phagocytosis and epidermal hyperproliferation would result in rapid clearance of *D.congolensis*.

In contrast, in the second situation the normal host immune response is inhibited by an array of external factors. Although the different susceptibilities of indigenous and exotic breeds (Leroy and Marchot 1987) suggest genetic factors may determine the outcome of infection, they do not have an overriding influence, as witnessed by the inability to induce chronic, generalised infections experimentally in any species or breed (Lloyd 1984). A chronic infection might arise either as a result of active immunosuppression, or because of a defect in some component of the immune response. Thus, mononuclear cells would still be attracted to the site of infection as a result of the inflammatory response induced by tissue damage but would not be activated because of a defect in antigen-presentation or subsequent cell-activation. Alternatively, T-suppressor cells would predominate following the induction of an immunosuppressive pathway. Although some pathways result in specific suppression of the

response to the inductive antigen, others cause a more general immunosuppressive effect (Granstein 1985). Indeed, Kimber, Pierce, Mitchell and Kinnaird (1987) demonstrated that a delayed hypersensitivity response to a contact-sensitising agent induced a transient suppression of the response to unrelated antigens.

Immunosuppression may arise as a result of a high biting-arthropod burden; studies of mite-infested animals, for example, have revealed decreased *in vitro* lymphocyte responses to mitogens (Corbett, Banks, Hinrichs and Bell 1975). Similarly, in tick infestations, suppression of mitogen-induced proliferation occurred and was concurrent with a positive lymphocyte transformation response to tick-antigen (Wikel and Osburn 1982). Wikel (1982a) found tick-infestation by *Dermacentor andersoni* caused a decreased lymphocyte response to the T-cell mitogens, concanavlin A and phytohaemagglutinin but not to the B-cell mitogen, lipopolysaccharide, suggesting that normal T-cell responses may be impaired during tick-exposure. Both ticks (Plowright 1956) and mites (Roberts and Vallely 1962) have been implicated in contributing to the occurrence of dermatophilosis.

The response to mitogens may reflect the overall ability of the lymphocytes to react to a stimulus but would not necessarily correlate with the response to an antigen since this requires the additional co-operation of other cell types. Fewer studies have been carried out on the response of arthropod-infested hosts to antigens other than those derived from the arthropod. However, Wikel (1985) demonstrated tick-induced suppression of the guinea-pig IgM response to the thymic-dependent antigen of sheep erythrocytes.

Davis and Philpott (1980) proposed that a *D.congolensis* lesion became chronic if initiated at the site of an arthropod bite because the DTH

reaction to the bite produced a local immunosuppression. They found that *D.congolensis* lesions persisted when initiated at the site of a DTH response to another agent.

Immunosuppression may also arise as a result of concurrent infections. Chronic schistosomiasis depressed the *in vitro* lymphocyte response to T-cell mitogens (Pelley, Ruffier and Warren 1976) as did the presence of *Leishmania tropica* (Farah, Lazary and De Weck 1976). In addition, *Trichinella spiralis*-infection suppresses the antibody response to other agents (Lubiniecki and Cypess 1975)

The occurrence of trypanosomiasis and rinderpest have often preceeded dermatophilosis (Plowright 1956). Furthermore, the normal cell-mediated response to the antigen PPD by rabbits immunised with Freund's complete adjuvant, was abrogated by *Trypanosoma congolense*-infection (Mansfield and Wallis 1974). The study demonstrated suppression of the *in vitro* lymphocyte response to PPD and to PHA, and of the PPD-stimulated production of MMIF and the *in vivo* DTH response, assessed by skin testing. In general, intercurrent infections are thought to depress cell-mediated responses (Turk 1981) and parasitised hosts have a decreased ability to respond immunologically to unrelated agents (Ogilvie and Wilson 1976).

A degree of general immunosuppression may be caused by increased levels of cortisone (Bach 1975)) induced by stress which may arise in response to various factors including the activity of biting arthropods. Malnutrition, another form of stress, has been incriminated in contributing to the persistence of *D.congolensis*-infection (Lloyd 1984).

Some skin diseases may progress to a chronic state when serum factors inhibit the cell-mediated response that would otherwise resolve the disease. Calderon and Hay (1984) found that the lymphoid cells of mice

suffering from a chronic *Trichophyton quinckeanum* infection conferred protection when transferred to recipient mice. However the adoptive immunity was abrogated when the cells were transferred along with autologous donor serum. The inhibitory serum factor was apparently dermatophyte antigen rather than antibody. Likewise, Fischer, Ballet and Griscell (1978) found that the serum of patients with chronic mucocutaneous candidiasis inhibited the patient's lymphocyte transformation response to *Candida albicans* and that the inhibition was caused by a *Candida* antigen in the serum. Thus, antigens from skin pathogens may enter the circulation and inhibit cell-mediated immunity. Jones and Artis (1981) suggested that immunological tolerance may develop in dermatophyte infections due to an overload of dermatophyte antigen. Similarly, Turk (1981) proposed that a high antigenic-load contributed to the lack of host resistance in the chronic skin diseases lepromatous leprosy, mucocutaneous candidiasis, diffuse leishmaniasis and secondary syphilis.

Animals in the field may be exposed to a constant high challenge by *D. congolensis* as a result of the multitudinous skin lesions caused by ticks, flies and thorny vegetation, all of which create potential entry sites for the bacterium. An abundance of *D. congolensis* antigen might then inhibit either the local or the systemic host immune response, giving rise to a chronic infection.

Several means by which the normal immune response to *Dermatophilus congolesis* may be depressed have been described. The suppressive factors may act locally, as shown by the prolonged *D. congolensis*-infections at the sites of DTH reactions to other agents, and/or exert a more general immunosuppressive effect, such as that provoked by intercurrent infections.

This study has identified some aspects of the host response to *Dermatophilus congolensis*, namely, the activation (Chapter 7) and proliferation (Chapter 2) of T-helper cells (Chapter 3) which secrete lymphokines capable of augmenting the immune response and possibly epidermal defence (Chapters 4 and 6). In particular, the localization and rapid resolution of the infection may be attributed, in part, to activated, antigen-specific T-helper cells. A defect in this activation might enhance the persistence and spread of *D.congolensis* giving rise to chronic, generalised dermatophilosis.

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The reference system used was that of Wilson (1966).
Abbreviations are those found in Index Medicus.

APPENDIX ONE

PURIFIED WATER

The water used for preparing media and other solutions for tissue culture was purified by the Milli Ro 60 system. It was then given a final polishing with the Milli Q system (Millipore), such that the resultant resistivity was 18 mega ohm cm^{-2}, indicating an extremely high purity.

STERILISATION OF MATERIALS FOR USE IN CELL CULTURE

Purified water was sterilised by heating to 121°C, under a pressure of 15 psi (1.1 kg cm^{-2}) for 15-20 minutes. Phosphate buffered saline (PBS) was also sterilised by this method. All other solutions were sterilised by passing through a 0.22 µm, low protein absorption, disposable filter (Millex GV, Millipore). Pipettes were sterilised in a dry heat oven at 170°C for 90 minutes; other glassware was sterilised by heating under a pressure of 15 psi for 15 minutes.

WASHING OF GLASSWARE FOR USE IN CELL CULTURE

All glassware used for tissue culture was washed by soaking in 0.5-1 percent Decon 75 (Decon Laboratories), rinsing thoroughly in tap water and then in purified water. Glass pipettes were washed as above except for extra washes in tap water.

PHOSPHATE-BUFFERED SALINE (PBS)

The Dulbecco A, calcium and magnesium-free formulation was used (Oxoid manual 1988).

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.20 g

Made up to 1 l with purified water.
All chemicals were anhydrous preparations of Analar grade material (BDH).
Final pH = 7.3 - 7.4

PENICILLIN STOCK SOLUTION

To a sterile $1x10^6$ unit vial of sodium benzyl penicillin (Glaxovet), 1.6 ml sterile, purified water was added, making a total volume of 2 ml. This was diluted by a factor of ten with 18 ml water, giving a stock solution of 50,000 units ml^{-1}. The stock solution was stored in 5 ml aliquots at -20°C and, once thawed, at 4°C. For use, depending on the type of culture, either 100 or 200 µl was added per 100 ml culture medium, giving a final concentration of 50 or 100 units ml^{-1} respectively.

STREPTOMYCIN STOCK SOLUTION

To a sterile one gramme vial of streptomycin sulphate (Evans) 1.2 ml sterile, purified water was added, making a total volume of 2 ml. This was diluted by a factor of ten with 18 ml water, giving a stock solution of 50 mg ml^{-1}. The stock solution was stored at -20°C, in 5 ml aliquots and, once thawed, at 4°C. For use, as with the penicillin stock, either 100 or 200 µl per 100 ml medium was added, giving a final concentration of 50 or 100 µg ml^{-1} respectively.

HEPARIN

Preservative-free heparin (Grade I, from porcine intestinal mucosa, Sigma) was dissolved in PBS to give a concentration of 1,000 units ml^{-1} (eg. 141 mg of a 177 units mg^{-1} preparation made up to 25 ml with PBS). The solution was passed through a 0.22 µm filter and stored at 4°C.

RPMI-1640 MEDIUM CONTAINING HEPARIN

To make 100 ml medium:

RPMI-1640 (Gibco)	97.0 ml
Heparin, 1,000 units ml^{-1} stock solution	0.2 ml
Foetal calf serum (Gibco)	2.0 ml
Penicillin, 50,000 units ml^{-1} stock solution	0.4 ml
Streptomycin sulphate, 50 mg ml^{-1} stock solution	0.4 ml

2-MERCAPTOETHANOL STOCK SOLUTION

2-Mercaptoethanol, with a molecular weight of 78, supplied as a solution in which 1 ml weighs 1.12 g (Sigma) was diluted 1,000x with PBS (1 ml made up to 1 l) to give a 14 mM solution. This was further diluted 2.8x (1ml to 1.8 ml PBS). This stock 5 mM solution was passed through a 0.22 µm filter and stored, in 5 ml aliquots at -20°C and, once thawed, at 4°C. For use, 1 ml of the stock solution was added to a total of 100 ml medium, giving a final culture concentration of $5x10^{-5}$ M.

COMPLETE RPMI-1640 MEDIUM

Media was stored at 4°C and used within three days of preparation. For 100 ml complete medium:

RPMI 1640, (containing 25 mM Hepes and 2 mM L-glutamine, Gibco)	89.6 ml
Myoclone foetal calf serum (Gibco)	8.0 ml
2-mercaptoetanol, 5 mM stock	1.0 ml
L-glutamine, 200 mM (Flow)	1.0 ml
Benzyl-penicillin, 50,000 units ml^{-1} stock	0.2 ml
Streptomycin sulphate, 50 mg ml^{-1} stock	0.2 ml

GIEMSA'S STAIN

Ten gram of Giemsa powder (Azur-eosin-methylene blue, Merck) was ground into 540 ml glycerol (Analar, BDH); the mixture was heated to 60°C for one hour with occassional shaking. Once cooled to room temperature, 840 ml methanol (Analar solvent methanol, BDH) was added and the mixture shaken vigorously, then stirred overnight. 2.75 g Azur II (Merck) was then added and the mixture stirred overnight again. This stock solution was filtered (Whatman no.4) and stored in a dark bottle. For use, a five per cent solution was made up in phosphate buffer (Gurr 6550, BDH) pH 7.2.

MONONUCLEAR CELL ISOLATION FROM BLOOD

Blood was withdrawn, direct from the heart of a rat anaesthetised with ether, into a sterile syringe containing 40 units ml^{-1} heparin. Mononuclear cells were isolated from the blood following the method of density gradient centrifugation described by Brown (1987). The blood was diluted with an equal volume of PBS and 20 ml was layered directly onto 8 ml Ficoll-paque . The gradient was centrifuged with a force of 720g at the interface between the Ficoll and the blood ($900g_{max}$, for 30 minutes at 15-20°C. The MC fraction was collected and washed twice in 20 ml volumes of PBS to remove platelets and Ficoll (300g for 10 min, then 300g for 5 min, 15-20°C). Finally, the cells were resuspended in

complete RPMI-1640 medium and aliquots taken for cell counts and cytocentrifuge preparations.

MONONUCLEAR CELL ISOLATION FROM SPLEEN

Yield of spleen mononuclear cells obtained from Ficoll gradients centrifuged at various forces.

G force (interface)	Median yield (%)	n
450	20	2
470	23	6
480	30	6
490	19	2
500	19	9

n represents the number of times carried out

PURIFIED PROTEIN DERIVATIVE OF *MYCOBACTERIUM TUBERCULOSIS* (PPD)

Preservative-free PPD was dissolved in PBS to give a concentration of 3 mg ml^{-1}. The solution was filter-sterilised and stored for up to a month at 4°C. For sensitisation of rats, 3 mg ml^{-1} PPD was emulsified with an equal volume of Freund's incomplete adjuvant (Difco). The emulsion was checked by ensuring that when a drop was placed on ice-cold water, it remained intact and did not disperse. For skin tests the solvent was sterile PBS and for use in SMC culture the solvent was complete RPMI medium.

BLOOD AGAR FOR GROWTH OF *D. CONGOLENSIS*

Blood agar (B/A) plates were prepared by dissolving 15.4 g Columbia agar base (Gibco) in 350 ml purified water, with boiling and frequent mixing. The solution was sterilised by heating under a pressure of 15 psi for 15 minutes. Once cooled to a temperature of 56°C, 25 ml of whole defibrinated sheep blood (Gibco) was added and gently shaken to mix. The blood agar was quickly poured into sterile petri dishes (Sterilin) and allowed to set. The plates were incubated for 24 hours at 37°C and checked for sterility, then stored at 4°C.

BROTH FOR GROWTH OF *D. CONGOLENSIS*

A 50/50 mixture of brain heart infusion and neutralised soya peptone (BHI/NSP, Oxoid) was prepared. Nineteen grams of each was dissolved in a total of one litre of purified water, by gentle heating. Aliquots of 15 ml were transferred to Universal bottles and sterilised by heating under a pressure of 15 psi for 15 minutes. For preparation of antigen, 400 ml volumes were made and autoclaved for an extra 5 minutes.

PREPARATION OF *D. CONGOLENSIS* ANTIGEN

Filament Antigen

A four-day, 400 ml, broth culture, checked for purity, was washed twice in PBS (1,000g, 10 min., 4°C) and the pellet resuspended in ten times its volume of purified water. The suspension was subjected to ultra-sound disintegration with twenty 45 second bursts of full amplitude (MSE 100 watt ultrasonic disintegrator, max. output 8 microns peak to peak). During this process, the suspension was kept on ice and occassionally mixed. The sonicated product was centrifuged at 2,300g for 30 minutes at 4°C and the supernatant collected and passed through a 0.22 µm Millex

filter unit (low protein absorption). The solution was lyophilised on a Virtis freeze-drier and then stored at -20°C under dessicating conditions

COCCI ANTIGEN

A suitable quantity of PBS was added to 48 hour B/A plate cultures of *D.congolensis* and the cocci harvested by gentle rubbing with a bent pasteur pipette. The suspension was left for one hour at 37°C to allow any filaments to settle out. After this time, the supernatant was collected and the antigen prepared from it in the same way as for the filament antigen.

VIABLE COUNTS FOR DETERMINATION OF *D.CONGOLENSIS* CELL NUMBERS

Serial, ten-fold, dilutions of the bacterial suspension were made up in sterile PBS, from 10^{-1} to 10^{-7}. Nine 25 µl aliquots of each dilution were dropped onto B/A plates, which were then incubated for 48 hours at 37°C. The mean colony count for each dilution was calculated and the initial cell concentration derived.

GRAM STAIN

Materials

Crystal Violet	0.5 per cent aqueous solution

Lugol's Iodine:

Iodine	1 g
Potassium iodide	2 g
Distilled water	100 ml

Carbol Fuschin: 5 per cent aqueous solution of Z.N. carbol fuschin

Z.N carbol fuschin:

Basic fuschin	1 g
Phenol	5 g
Ethanol	100 ml
Distilled water	100 ml

Dissolve basic fuschin in phenol with gentle heating, then add ethanol and water.

Acetone

The crystal violet, Lugol's iodine and carbol fuschin solutions were filtered (Whatman no. 1) before use.
All materials were obtained from BDH.

Method

Smears, from cultures, were prepared on clean glass slides and allowed to air dry. The smears were then heat-fixed and bathed in crystal violet for 30 seconds, followed by Lugol's iodine for 30 seconds and acetone for 2 seconds. The acetone was washed off with tap water and the smear bathed in carbol fuschin for 30 seconds.

APPENDIX TWO

HANKS BALANCED SALT SOLUTION-BOVINE SERUM ALBUMIN (HBSS-BSA)	
HBSS (10 x) (Gibco)	10 ml
Purified water	85 ml
Sodium bicarbonate, 7.5 % (Gibco)	3 ml
Hepes buffer, 1 M (Gibco)	2 ml
Bovine serum albumin (Sigma)	1 g

FIXATIVE FOR INDIRECT FLUORESCENT-ANTIBODY TEST	
Formaldehyde, 40 per cent Analar grade (BDH)	10 ml
Glucose, Analar grade (BDH)	2 g
PBS	90 ml

APPENDIX THREE

HANK'S BALANCED SALT SOLUTION / HEPARIN

For each 100 ml of solution, the following additions were made:

Hank's balanced salt solution, (10 x) (Gibco)	10.0 ml
Purified water	86.6 ml
Sodium bicarbonate, 7.5 % solution (Gibco)	0.5 ml
Hepes buffer, 1 M solution (Gibco)	2.0 ml
Heparin, 1,000 units ml^{-1} stock (Appendix 1)	0.5 ml
Penicillin, 50,000 units ml^{-1} stock (Appendix 1)	0.2 ml
Streptomycin sulphate, 50 mg ml^{-1} (Appendix 1)	0.2 ml

EAGLE'S MINIMAL ESSENTIAL MEDIUM

Eagle's MEM, with Earle's salts (10 x) (Gibco)	10.0 ml
Purified water	83.6 ml
Foetal calf serum (heat inactivated) (Gibco)	10.0 ml
Sodium bicarbonate, 7.5 %	3.0 ml
Hepes buffer, 1 M	2.0 ml
L-glutamine, 200 mM (Flow)	1.0 ml
Penicillin, 50,000 units ml^{-1}	0.2 ml
Streptomycin sulphate, 50 mg ml^{-1}	0.2 ml

The pH of the medium always lay between 7.2 and 7.4.

Individual migration distances corresponding to the median values in table 4.1

Expt.	Migration distance in presence of test supernatant (units)	Migration distance in presence of control supernatant (units)
1	80,50,30,20,20,30,20	70,85,35,90,40,70,35
2	50,30,45,65,35,60,40,25, 25,25,35,25,60,	55,55,55,50,80,55,55
3	30,45,45,55,60,55,55,60,70,20	70,65,70,50,70,60,80
4	45,40,55,55,65,70,50	80,105,90,70,55,80
5	50,50,55,60,50	100,90,90,80,80

Distances were measured using an eyepiece micrometer scale, to the nearest 5 units, where 1 unit is equivalent to 20 μm.

Individual migration distances corresponding to the median values in table 4.2

Expt.	Migration distance in presence of test supernatant (units)	Migration distance in presence of control supernatant (units)
1	50,65,50,40,40,70,30,50	70,50,65,70,65,25,50
2	70,75,85,70,60,65,65,80,80,75	60,70,50,80,65,75,60,80,75,80
3	55,80,50,40,80,50,25,20	80,50,55,40,35,75,75,60,60,35

Distances were measured using an eyepiece micrometer scale, to the nearest 5 units, where 1 unit is equivalent to 20 μm.

APPENDIX FOUR

INCOMPLETE EAGLE'S MINIMAL ESSENTIAL MEDIUM (MEM)

Incomplete MEM was made up as required. For each 100ml of solution, the following were added:

Eagle's MEM with Earle's salts, 10 x	10.0 ml
Purified water	84.6 ml
Sodium bicarbonate, 7.5 %	3.0 ml
Hepes buffer, 1M	2.0 ml
Penicillin, 50,000 units ml^{-1} (Appendix 1)	0.2 ml
Streptomycin, 50 mg ml^{-1} (Appendix 1)	0.2 ml

TRYPSIN IN MEM

Trypsin (1:250, Difco) was dissolved in incomplete MEM to give a 0.25 per cent solution; this was filtered through a low protein absorption 0.22 µm filter twice, since mycoplasmas sometimes occur in commercial trypsin preparations and are not readily removed by a single 0.22 µm filtration. The solution was stored at 4°C for up to one month.

DISPASE

Grade two dispase (neutral protease, Boehringer Manheim) was dissolved in incomplete MEM to give a 0.2 per cent solution. This concentration is equivalent to 1 unit ml^{-1} (Boehringer Manheim 1988 catalogue). The solution was then passed through a 0.22 µm low protein absorption filter and stored at 4°C for up to one month.

TRYPSIN IN EDTA

The trypsin solution used for disaggregating basal cells from epidermal strips, was made by dissolving 0.2 g trypsin in 100 ml of 0.02 per cent ethylenediaminetetracetic acid (EDTA, Sigma) in PBS. The solution was filtered twice, 0.22 µm low protein absorption, and stored at 4°C for up to one month.

PERCOLL GRADIENT

The four densities of Percoll (Pharmacia) were prepared by the following additions:

Density ($g\ ml^{-1}$)	Percoll (ml)	Purified water (ml)	1.5 M Sodium chloride (ml)
1.01	0.25	6.95	0.80
1.03	1.48	5.72	0.80
1.06	3.32	3.88	0.80
1.09	5.17	2.03	0.80

The sodium chloride was Analar grade (BDH), made up in purified water and autoclaved.

COMPLETE S-MEM MEDIUM (LOW CALCIUM-CONCENTRATION)

Each 100 ml of medium contained the following:

S-MEM, 10 x (Gibco)	10.0 ml
Purified water	85.3 ml
Sodium bicarbonate, 7.5 %	1.0 ml
Hepes buffer, 1M	2.0 ml
FCS, Myoclone (replaces 5 ml of incomplete media)	5.0 ml
L-glutamine, 200 mM	1.0 ml
Hydrocortisone, 80 $\mu g\ ml^{-1}$ (Sigma)	0.5 ml
Penicillin, 50,000 units ml^{-1}	0.1 ml
Streptomycin, 50 $\mu g\ ml^{-1}$	0.1 ml

COMPLETE S-MEM (NORMAL CALCIUM-CONCENTRATION)

The medium was the same as S-MEM complete medium, except that 1 ml of sterile 17.6 g Ca $Cl_2.2H_2O$ l^{-1} solution (Analar, BDH) replaced 1 ml of purified water, giving a calcium ion concentration of 1.2 mM. The final calcium concentration was 1.4 mM, since the FCS contains 4.6 mM calcium.

COMPLETE M199 MEDIUM

Each 100 ml of complete medium contained the following:

M199 media, 10 x (Gibco)	10.0 ml
Purified water	85.3 ml
Sodium bicarbonate, 7.5 %	1.0 ml
Hepes buffer, 1 M	2.0 ml
FCS, Myoclone (replaces 13 ml of incomplete media)	13.0 ml
L-glutamine, 200 mM	1.0 ml
Hydrocortisone, 80 $\mu g\ ml^{-1}$	0.5 ml
Penicillin, 50,000 units ml^{-1}	0.1 ml
Streptomycin, 50 mg ml^{-1}	0.1 ml

EPIDERMAL GROWTH FACTOR

EGF from mouse submaxillary gland (Sigma) was dissolved in incomplete MEM to give a 10 $\mu g\ ml^{-1}$ solution. The solution was passed through a low protein absorption 0.22 μm filter and stored in aliquots at -20°C. For use, 0.1 ml was added to 100 ml of media to give a 10 ng ml^{-1} solution.

INSULIN

Insulin from bovine pancreas (Sigma) was dissolved in purified water, acidified to pH 4 by the addition of 0.2 N hydrochloric acid (Analar, BDH). The 5 mg ml^{-1} solution was passed through a low protein absorption 0.22 μm filter and stored in aliquots at -20°C. For use, 0.1 ml was added to 100 ml of media to give 5 $\mu g\ ml^{-1}$.

CHOLERA TOXIN

Cholera toxin, supplied lyophilised in buffer (Sigma), was dissolved in purified water to give a 0.5 mg ml^{-1} solution. This was diluted 50 times with PBS, to 10 $\mu g\ ml^{-1}$ and the solution passed through a low protein absorption 0.22 μm filter. The stock was stored at 4°C. For use, 0.1 ml was added to 100 ml of media to give 10 ng ml^{-1}.

TRANSFERRIN

Rat transferrin (Sigma), supplied lyophilised in 1 mg aliquots, was dissolved as required in 200 ml incomplete media. The solution was passed through a low protein absorption, 0.22 μm filter. The culture concentration was 5 $\mu g\ ml^{-1}$.

APPENDIX FIVE

COMPLETE S-MEM MEDIUM (LOW CALCIUM CONCENTRATION)

Each 100 ml of media contained the following:

S-MEM, 10 x	10.0 ml
Purified water	85.3 ml
Sodium bicarbonate, 7.5 %	1.0 ml
Hepes buffer, 1M	2.0 ml
FCS, Myoclone (replaces 5 ml of incomplete media)	5.0 ml
L-glutamine, 200 mM	1.0 ml
Hydrocortisone, 80 $\mu g\ ml^{-1}$ (Sigma)	0.5 ml
Cholera toxin, 10 $\mu g\ ml^{-1}$	0.1 ml
Penicillin, 50,000 units ml^{-1}	0.1 ml
Streptomycin, 50 $\mu g\ ml^{-1}$	0.1 ml

COMPLETE S-MEM MEDIUM (NORMAL CALCIUM CONCENTRATION)

The medium was the same as S-MEM complete medium, except that 1 ml of sterile 17.6 g Ca $Cl_2.2H_2O$ l^{-1} solution (Analar, BDH) replaced 1 ml of purified water, giving a calcium ion concentration of 1.2 mM. The final calcium concentration was 1.4 mM, since the FCS contains 4.6 mM calcium.

Experiment one: Median CPM values for epidermal cells in low calcium medium in the presence of SMC supernatants from infected rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0)	4 (3-4)	0 (0)	18 (16-21)
1/10	2 (2-3)	8 (7-9)	11 (10-12)	19 (17-21)
1/100	8 (7-10)	10 (9-10)	18 (15-25)	21 (18-22)
1/1,000	8 (8-9)	10 (9-10)	19 (16-20)	23 (22-25)
Control supernatant				
1/2	0 (0)	8 (6-10)	0 (0)	39 (33-41)
1/10	5 (4-7)	11 (10-12)	17 (15-18)	30 (27-35)
1/100	4 (3-6)	9 (8-10)	22 (18-23)	24 (23-26)
1/1,000	7 (6-8)	8 (7-9)	22 (20-23)	22 (21-23)
Medium alone	13 (9-14)		17 (16-18)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 88.

Experiment two: Median CPM values for epidermal cells in low calcium medium in the presence of SMC supernatants from infected rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0)	2 (2-4)	0 (0)	20 (19-22)
1/10	1 (1-2)	4 (4-5)	7 (6-10)	21 (20-23)
1/100	4 (4-5)	4 (4-5)	14 (14-15)	17 (16-18)
1/1,000	5 (4-6)	4 (3-5)	14 (13-15)	16 (15-17)
Control supernatant				
1/2	0 (0)	7 (6-7)	0 (0)	41 (35-44)
1/10	1 (0)	5 (5-6)	8 (7-9)	25 (24-26)
1/100	3 (3-4)	4 (4-5)	15 (14-17)	15 (14-17)
1/1,000	4 (4-5)	4 (0)	16 (14-18)	15 (14-15)
Medium alone	4 (3-5)		13 (12-14)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 130.

Experiment three: Median CPM values for epidermal cells in low calcium medium in the presence of SMC supernatants from infected rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0)	1 (1-2)	1 (0)	9 (8-12)
1/10	1 (1-2)	3 (2-3)	11 (8-12)	14 (12-16)
1/100	4 (3-5)	4 (3-4)	17 (16-20)	16 (15-18)
1/1,000	5 (4-5)	5 (4-6)	19 (18-20)	18 (17-20)
1/10,000	6 (3-6)	5 (5-6)	19 (18-21)	18 (17-19)
Control supernatant				
1/2	0 (0)	4 (3-4)	1 (0)	25 (20-30)
1/10	1 (1-2)	5 (4-6)	12 (11-13)	24 (22-26)
1/100	4 (3-4)	4 (3-4)	17 (16-20)	19 (18-20)
1/1,000	4 (3-4)	4 (3-5)	19 (18-20)	17 (16-19)
1/10,000	4 (3-5)	5 (4-5)	20 (18-21)	18 (17-20)
Medium alone	4 (3-4)		17 (15-18)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 15.

Experiment four: Median CPM values for epidermal cells in low calcium medium in the presence of SMC supernatants from naive rats

	Median CPM x 10^{-3}			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0)	1 (0-1)	0 (0)	11 (11-14)
1/10	0 (0)	0 (0)	5 (4-6)	7 (6-9)
1/100	0 (0-1)	1 (0-1)	10 (10-11)	9 (8-10)
1/1,000	1 (0-1)	1 (0-1)	13 (12-13)	11 (9-12)
Control supernatant				
1/2	0 (0)	1 (1-2)	0 (0-1)	17 (15-19)
1/10	0 (0)	1 (0)	6 (6-7)	13 (11-14)
1/100	0 (0)	0 (0-1)	10 (9-11)	10 (8-10)
1/1,000	1 (0-1)	1 (0-1)	12 (11-14)	12 (10-14)
Medium alone	1 (0-1)		12 (11-13)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 40.

Experiment five: Median CPM values for epidermal cells in low calcium medium in the presence of SMC supernatants from naive rats

	Median CPM x 10^{-3}			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	2 (1-2)	4 (3-5)	3 (3-4)	11 (7-12)
1/10	6 (5-7)	7 (6-9)	18 (16-19)	17 (14-23)
1/100	6 (5-8)	7 (6-8)	18 (16-20)	15 (11-17)
1/1,000	6 (6-7)	8 (7-9)	17 (14-20)	15 (12-19)
Control supernatant				
1/2	1 (1-2)	7 (5-8)	4 (2-4)	22 (15-27)
1/10	7 (6-9)	8 (7-10)	23 (21-27)	22 (18-26)
1/100	7 (6-8)	7 (6-8)	18 (17-21)	16 (13-19)
1/1,000	7 (6-9)	7 (6-8)	19 (15-20)	15 (13-18)
Medium alone	7 (5-8)		16 (14-20)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 430.

Experiment six: Median CPM values for epidermal cells in low calcium medium in the presence of SMC supernatants from naive rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0)	2 (1-3)	2 (2-3)	7 (5-9)
1/10	3 (2-4)	4 (3-4)	13 (12-15)	9 (9-10)
1/100	4 (3-5)	4 (0)	13 (12-15)	11 (8-14)
1/1,000	5 (4-6)	5 (4-6)	15 (13-17)	12 (11-17)
Control supernatant				
1/2	1 (0)	5 (4-6)	3 (0)	23 (17-27)
1/10	5 (4-6)	5 (5-6)	20 (18-24)	19 (16-21)
1/100	4 (3-4)	4 (4-5)	13 (12-15)	15 (12-18)
1/1,000	4 (3-5)	5 (4-6)	16 (15-18)	15 (13-17)
Medium alone	5 (3-7)		15 (11-18)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 520.

Experiment seven: Median CPM values for epidermal cells in normal calcium medium in the presence of SMC supernatants from infected rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0-1)	5 (5-6)	1 (0)	10 (9-11)
1/10	2 (0)	4 (4-5)	5 (0)	9 (8-9)
1/100	6 (5-6)	5 (5-6)	9 (8-9)	9 (8-9)
1/1,000	6 (6-7)	6 (6-7)	9 (8-10)	9 (8-10)
Control supernatant				
1/2	0 (0)	9 (8-10)	0 (0)	25 (23-29)
1/10	1 (1-2)	6 (6-8)	5 (4-5)	14 (13-17)
1/100	5 (5-6)	6 (5-6)	9 (8-13)	10 (9-11)
1/1,000	6 (5-6)	6 (5-6)	9 (8-9)	10 (10-11)
Medium alone	6 (6-7)		9 (8-10)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 50.

Experiment eight: Median CPM values for epidermal cells in normal calcium medium in the presence of SMC supernatants from infected rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0)	11 (10-11)	2 (1-2)	20 (19-22)
1/10	3 (3-4)	10 (9-10)	7 (6-8)	13 (12-14)
1/100	8 (7-9)	10 (9-10)	10 (8-11)	11 (10-12)
1/1,000	8 (7-9)	10 (9-10)	10 (9-11)	11 (10-13)
Control supernatant				
1/2	0 (0)	11 (10-13)	1 (0)	21 (14-23)
1/10	3 (3-4)	11 (10-12)	7 (6-8)	14 (10-16)
1/100	8 (8-10)	10 (9-10)	10 (9-11)	11 (7-12)
1/1,000	8 (8-10)	10 (9-10)	10 (9-11)	11 (9-12)
Medium alone	8 (7-9)		10 (9-11)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 60.

Experiment nine: Median CPM values for epidermal cells in normal calcium medium in the presence of SMC supernatants from naive rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	1 (0)	25 (23-27)	0 (0)	35 (29-38)
1/10	6 (4-6)	10 (8-11)	11 (10-12)	31 (30-33)
1/100	6 (5-7)	7 (0)	20 (18-22)	24 (17-25)
1/1,000	6 (4-6)	6 (0)	20 (17-20)	22 (18-23)
Control supernatant				
1/2	2 (1-2)	27 (19-28)	0 (0)	40 (36-43)
1/10	5 (4-5)	8 (6-10)	14 (8-14)	36 (30-39)
1/100	6 (5-6)	7 (6-8)	19 (18-21)	24 (14-28)
1/1,000	5 (5-6)	7 (4-7)	19 (18-21)	21 (19-24)
Medium alone	5 (4-6)		5 (5-6)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 120.

Experiment ten: Median CPM values for epidermal cells in normal calcium medium in the presence of SMC supernatants from naive rats

	Median CPM x 10^3			
Dilution Time (h)	24		48	
	Dialysed	Non-dialysed	Dialysed	Non-dialysed
Stimulated supernatant				
1/2	0 (0-1)	14 (12-15)	0 (0)	24 (22-28)
1/10	3 (3-4)	6 (3-7)	8 (7-8)	23 (22-25)
1/100	6 (4-6)	5 (3-5)	16 (14-18)	18 (16-19)
1/1,000	6 (3-6)	5 (3-6)	17 (16-19)	15 (14-17)
Control supernatant				
1/2	0 (0)	16 (10-21)	0 (0)	30 (27-33)
1/10	3 (3-4)	6 (2-7)	11 (10-12)	30 (28-33)
1/100	6 (4-6)	5 (3-5)	16 (15-18)	21 (19-22)
1/1,000	6 (4-7)	6 (4-6))	17 (16-18)	19 (17-20)
Medium alone	5 (3-6)		17 (15-19)	

Values are medians of ten replicates, rounded to the nearest 1,000, with the range in brackets. The times refer to time after addition of supernatant. The median CPM value at time 0 was 80.

APPENDIX SIX

CHROME ALUM/GELATIN SOLUTION

Gelatin (Oxoid) was dissolved in purified water to give a 0.5 per cent solution. Chromium alum (BDH) was then dissolved in the gelatin solution to a final concentration of 0.05 per cent. The solution was stored at 4°C.

NRS-BSA-BUFFER

Tween 80 (BDH) was dissolved in PBS to give a 0.08 per cent solution, which was then supplemented with two per cent normal rat serum (NRS) and 0.5 per cent bovine serum albumin (BSA). The buffer was prepared on the day of use.

PEROXIDASE CONJUGATE

The rabbit anti-mouse IgG peroxidase conjugate was supplied in 250 µg quantities (Serotec). The conjugate was diluted in one per cent BSA in PBS to a total volume of 1 ml. This 250 µg ml^{-1} solution was stored in aliquots at -20°C. For use, the stock was diluted by a factor of 25 with NRS-BSA-buffer to give a 10 µg ml^{-1} solution which was stored at 4°C for up to one week. The conjugate was never refrozen.

DIAMINOBENZIDINE SOLUTION (DAB)

A 10 g vial of DAB (3,4-3,4 Tetra-aminobiphenyl hydrochloride, BDH) was dissolved in 15 ml PBS. The solution was filtered (Whatman no. 1) and 40 µl of hydrogen peroxide added just before use.

OSMIUM TETROXIDE

A 5 ml vial of four percent osmium tetroxide (BDH) was dissolved in PBS to give a final concentration of 0.1 per cent. The solution was stored in the dark at 4°C and discarded as soon as it became discoloured.

MAYER'S HAEMATOXYLIN

Haematoxylin	2.0 g
Sodium iodate	0.2 g
Ammonium alum	50.0 g
Distilled water	1.0 l

Leave overnight, then add the following:

Chloral hydrate	50.0 g
Citric acid	1.0 g

Boil for five minutes, cool and filter (Whatman no.1)

All chemicals from BDH.

SCOTT'S TAP WATER SUBSTITUTE

Sodium bicarbonate	7.0 g
Magnesium sulphate	40.0 g
Tap water	2.0 l

Chemicals from BDH.

PUTT'S EOSIN

Eosin Y	70 g
Potassium dichromate	35 g
Saturated aqueous picric acid	700 ml
Absolute ethanol	700 ml
Distilled water	700 ml

All chemicals from BDH

APPENDIX SEVEN

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